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1 OCTOBER 1984

Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D.C. 20307–5100

SUMMARY

THE VARIOUS SUBJECTS COVERED IN THIS REPORT ARE LISTED IN THE TABLE OF CONTENTS. ABSTRACTS OF THE INDIVIDUAL INVESTIGATIONS ARE INCLUDED ON THE DD FORM 1498 INTRODUCING EACH WORK UNIT REPORT, AND NAMES OF THE INVESTIGATORS ARE GIVEN AT THE BEGINNING OF EACH REPORT.

MISSION

1. The mission of the Walter Reed Army Institute of Research is to provide general military medicine research capability and conduct research in the areas of communicable diseases and immunology, combat surgery, combat psychiatry, drug development, medicine, military hazards of blast overpressure, chemical and nuclear warfare defense, and the biological effects of microwave irradiation; plan and conduct graduate education programs as directed; and conduct undergraduate level training programs to provide to the Army Medical Department personnel experienced and trained in military medical research capabilities.

2. Under the provisions of AR 40-5 and AR 40-441, WRAIR will:

a. Provide epidemiologic consultation services for the Office of The Surgeon General and other agencies.

b. Provide advisory services on problems in procedures or techniques in military medicine.

c. Provide special technical quality control where required, and conduct development studies in relation to biological products presenting problems of military importance.

d. Act as a diagnostic reference source for difficult medical problems and evaluations that require complicated analyses or tests not available in other Army installations.

e. Provide technical supervision of extramural contracts and grants sponsored by the U.S. Army Medical Research and Development Command that are specifically related to WRAIR in-house programs.

f. Provide command, control, and technical supervision and support for the U.S. Army Medical Research Institute of Infectious Diseases and the Walter Reed Army Institute of Research Special Foreign Activities.

FOREWORD

IN CONDUCTING THE RESEARCH DESCRIBED IN THIS REPORT, THE INVESTIGATORS ADHERED TO THE "GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS" AS PREPARED BY THE COMMITTEE ON CARE AND USE OF LABORATORY ANIMALS OF THE INSTITUTE OF LABORATORY ANIMAL RESOURCES, NATIONAL RESEARCH COUNCIL.

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Veterinary Medicine
Rickettsial Diseases
Military Stress

(OVER)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigators are included on the DD Form 1498 introducing each work unit report.

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PROJECT 3A161101A91C

IN-HOUSE LABORATORY INDEPENDENT RESEARCH

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11. TITLE *(Precede with Security Classification Code)* (U) Mechanisms of Human Mononuclear Cells for Killing of Intracellular Parasites

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0613 Microbiology 0603 Biology

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c. TELEPHONE NUMBER (include area code) 202 576-3551	d. TELEPHONE NUMBER (include area code) 202 576-3544
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22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Mononuclear cells; (U) In Vitro; (U) Leishmania; (U) Intracellular Killing

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The objective of this work unit is to elucidate the mechanisms of protective immunity to leishmania. Leishmaniasis is endemic in Africa, the Mid East and Indian Subcontinent and South America, posing a significant potential threat to military operations in these areas.

24. (U) The approach is to cultivate human monocytes in vitro, infect them with Leishman examine the processes of entry into the cell and intracellular replication, and examine the effect of immunomodulatory agents on these processes.

25. (U) 83 10 - 84 09 We have infected normal human blood monocytes with amastigotes of L. donovani and L. tropica and documented parasite replication. We have treated monocytes with interferon-containing lymphokines derived from mitogen-stimulated lymphocyte cultures. Human monocytes from normal subjects kill amastigotes of L. donovani, but not those of L. donovani, but not those of L. tropica after treatment with lymphokines. This observation suggests that these two pathogenic parasite species are killed by different mechanisms. We have also noted at least 2 distinct lymphokines that activate monocytes to kill L. donovani. One of these is interferon-gamma. The other is a 25-30,000 MW factor that is not neutralize by antibody to interferon and differs from interferon in its sensitivity to heating. This novel factor is potentially interesting as an immunotherapeutic molecule that could enhance the ability of human cells to kill Leishmania and enhance recovery from leishmaniasis. We a purifying and biochemically characterizing this substance to further detailed investigation. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oc 83 - 30 Sep 1984.

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PROJECT 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 095 Mechanisms of Human Mononuclear Cells for Killing of Intracellular Parasites

Investigators:

Principals: MAJ David L. Hoover, MC
LTC Wayne T. Hockmeyer, MSC

Associates: MAJ Monte S. Meltzer, MC
CPT Micheal J. Gilbreath, MSC
SP4 David Wynn

Problems and Objectives:

Leishmania species cause skin and visceral disease throughout the tropics. No effective strategy has been developed to prevent acquisition of disease by troops deployed in endemic areas. In animal models, cellular immunity is believed to control established disease. Murine macrophages activated by products of stimulated lymphocytes *in vitro*, for example are resistant to infection by *Leishmania* and destroy those parasites that do enter the host cell. If similar processes govern human macrophage-parasite interactions, enhancement of human host defenses by immunomodulating agents such as gamma interferon might be possible. Construction of an effective immunotherapeutic strategy requires the development of a system to allow analysis of the interplay between immuno-stimulated human monocytes-macrophages and parasites. The following problems must be addressed in the design of such a system: (1) What factors regulate parasite entry into the host cell? (2) Can human monocytes support intracellular replication of the parasite *in vitro*? (3) If so, can monocytes respond to immune mediators, especially interferons, to inhibit such replication and kill intracellular parasites? Evidence from the murine system indicates that immature mononuclear phagocytes are readily infected with *Leishmania* and support its replication, but respond poorly to immune mediators to kill it. Mature macrophages, however are responsive. (4) If human monocytes also prove to be relatively refractory to activation by immunomodulators to kill intracellular parasite, can such refractoriness be overcome by providing additional priming signals or by targeting mediators to the cell (e.g., by encapsulation in liposomes or by attachment of mediators to monocyte-specific antibody)?

Progress:

We have separated monocytes from normal human blood and exposed them to leishmania amastigotes. Amastigotes of both L. tropica and L. donovani infect and replicate in freshly harvested human monocytes. Monocytes treated with human gamma interferon kill L. donovani but not L. tropica amastigotes. These findings suggest that different microbicidal mechanisms are required to kill different species of Leishmania. The induction of microbicidal activity against L. donovani by interferon was dose related; was maximal when parasites and interferon were added to monocytes simultaneously; and was inhibited by the presence of anti-interferon antibody. Lymphokines (supernatants of mitogen-stimulated lymphocyte cultures) also induced microbicidal activity against L. donovani. Dose-response experiments suggested that lymphokine-induced microbicidal activity was greater than expected for the measured levels of interferon activity. In further experiments, lymphokine-induced microbicidal activity was not removed by antibody to interferon; appeared in regions of 30,000 and 50,000 molecular weight on gel chromatography; was resistant to heat inactivation; and was not inhibited by polymixin B. These findings suggest that supernatants of mitogen-stimulated human leukocyte cultures contain a human monocyte activating factor distinct from gamma interferon. This factor, because it induces antimicrobial activity, may be useful as an immunomodulating agent to treat established infections or to prevent acquisition of infection by exposed troops.

Recommendations:

We will investigate the following problems in activation of human monocytes to kill Leishmania: (1) What is the nature of the lymphokine that induces anti-leishmanial activity in human monocytes? We will approach this problem by producing monoclonal antibodies with partially purified preparations of lymphokine; screening genomic libraries for expression of the activity; and production of the lymphokine by recombinant DNA technology. (2) Can the application of different signals or alterations in the timing or mechanism of delivery of signals (e.g., by liposome encapsulation enhance the responsiveness of monocytes? (3) Can more mature or more differentiated human macrophages kill Leishmania after lymphokine treatment? (4) Do humoral factors (antibody and complement) enhance lymphokine-induced microbicidal activity?.

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3. Meltzer, M. S.: Macrophages as regulatory cells for tissue integrity during steady-state and inflammation. *J. Assoc. Mil. Derm.*

4. Gilbreath, M. J., Nacy, C. A., Hoover, D. L., Alving, C. R., Swartz, G. M. Jr. and Meltzer, M. S.: Differential inhibition of macrophage microbicidal activity by liposomes. Infect. Immunol.

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c. CONTRIBUTING	None			

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a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	2.0	39
85	2.0	40

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of CD&I
b. ADDRESS *(include zip code)* Washington, D C 20307 5100	b. ADDRESS Walter Reed Army Institute of Research Washington, DC 20307 5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Seid, R Jr
d. TELEPHONE NUMBER *(include area code)* 202 576-3551	d. TELEPHONE NUMBER *(include area code)* 202 576-3303
21. GENERAL USE - FINA MILITARY CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Formal, S B g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Kopecko, D J

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Enteric diseases; (U) O-specific Polysaccharide; (U) Lipopolysaccharide; (U) Recombinant DNA

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23(U) Development of nontoxic O-specific polysaccharide (O-Ps) antigens as immunizing agents against infections of military importance.

24(U) O-Ps antigens will be isolated from bacteria defective in lipopolysaccharide (LPS) synthesis or from recombinants infected with DNA genes encoding O-Ps synthesis. Recombinants expressing the O-Ps antigens will be detected by replica blot immunoassays. The O-Ps will be isolated, purified, and characterized by biochemical and analytical techniques. The toxicity will be measured by the Limulus assay. Non-toxic antigens will be tested for immunogenicity in laboratory animals.

25(U) 83 10-84 09 A Salmonella-Shigella transconjugant was found to elaborate both a lipid-A free Salmonella O-Ps and a Shigella O-Ps in addition to regular Salmonella LPS antigens. On a gel filtration column, the Salmonella O-Ps, eluted behind the Shigella O-Ps antigen, thus indicative of a smaller size. Both of these O-Ps antigens, isolated in 0.12-0.14 percent yield, were 1000 fold less toxic than native LPS in the Limulus assay. Chemical analyses revealed the Salmonella O-Ps as a repeating tetrasaccharide while the Shigella O-antigen as a dissacharide repeat unit. Physicochemical data suggest the reducing end of the O-Ps antigens containing a phosphorylated lipid carrier responsible for aggregation and slow electrophoretic mobility. Immunological testing, so far, indicates that the nontoxic Shigella O-Ps antigen, despite its large size, is poorly immunogenic in mice as compared to native LPS. (For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 to 30 Sept 84).

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 96: Immunochemistry of Non-toxic O-specific Polysaccharide Antigens

Investigators:

Principal:	Robert C. Seid, Jr., Ph.D.
Associates:	Samuel B. Formal, Ph.D. Louis S. Baron, Ph.D. Dennis J. Kopecko, Ph.D.

Objectives

The objectives are (1) to isolate and to purify lipid A free, O-specific polysaccharide (O-Ps) antigens from hybrid enteric organisms, (2) to compare their physicochemical properties with parental lipopolysaccharide (LPS) antigens, and (3) to determine their immunogenicity in laboratory animals.

Progress

A *S. typhi-Shigella* transconjugant (galactose epimeraseless), harboring a *Shigella* plasmid, when grown in the presence of galactose, was found to elaborate both a lipid-A free *S. typhi* O-specific polysaccharide (O-Ps) and a *Shigella sonnei* O-Ps in addition to regular *S. typhi* LPS antigens. On a Sepharose 4B filtration column in disaggregating buffer, the *S. typhi* O-Ps, eluted behind the *Shigella* O-Ps antigen, thus indicative of a smaller size. Both these O-Ps antigens, isolated in 0.12-0.14% yield, were 1000 fold less toxic than native lipopolysaccharides in the *Limulus* assay. Chemical analyses revealed the *S. typhi*O-Ps as a repeating tetrasaccharide composed of tyvelose, rhamnose, mannose, and galactose, while the Shigella O-antigen as a disaccharide repeat unit, composed of two unique amino sugars. Physiocochemical data indicate that the reducing end of the O-Ps antigen in the transconjugant strain is associated with a phosphorylated lipid carrier that is responsible for aggregation and slow electrophoretic mobility on SDS gels. In contrast to current dogma of LPS assembly, these O-Ps antigens are transported to the outer membrane without covalent linkage to core-lipid A, and exist as polymerized, antigenic surface entities. Immunological testing, so far, indicates that the nontoxic S. sonnei O-Ps antigen, despite its large size, is poorly immunogenic in mice as compared to native and toxic S. sonnei lipopolysaccharides.

Future Plans

The isolation, purification, and immunochemical characterization of other polysaccharide antigens resulting from genetic transfer of plasmids and DNA genes to other defined mutants of enteric bacteria are ongoing projects. This long term project should furnish additional insights into the mechanism of LPS assembly as well as to aid in the development of other genetically derived multivalent bacterial vaccine strains.

Bibliography

1. Seid, R.C., Jr., D.J. Kopecko, J.C. Sadoff, H. Schneider, L.S. Baron, and S.B. Formal. (1984). Unusual Lipopolysaccharide Antigens of a *Salmonella typhi* Oral Vaccine Strain Expressing the *Shigella sonnei* Form I Antigen. J. Biol. Chem. 259:9028-9034.

2. Schneider, H., T.L. Hale, W.D. Zollinger, R.C. Seid, Jr., C.A. Hammack, and J.McL. Griffiss. (1984). Heterogeneity of molecular size and antigenic expression within lipooligosaccharides of individual strains of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Infect. Immun. 45:544-549.

3. Wachter, R., R.C. Seid, Jr., L. Briggs, S. Bondarew, and E. Stevenson. (1984). Preparation and characterization of phase I antigen of *Coxiella burnetti*, manuscript submitted for publication.

4. Baron, L.S., D.J. Kopecko, S.B. Formal, P. Guerry, L. Hale, R. Seid, O. Washington, and C.A. Life. (1984). Construction of a fused plasmid specifying *Shigella flexneri* 2a antigens and transfer of this plasmid to the *Salmonella typhi* Ty21a *galE* oral vaccine strain. Abstract, 84th annual meeting of the Amer. Soc. Microbiol.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA 300521	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	97 WWGU
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) Immunochemistry on Binding Domain Peptides of Bacterial Pili

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE 82 10	14. ESTIMATED COMPLETION DATE Cont	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	25
85	2.0	28

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of CD&I
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Seid, R Jr
d. TELEPHONE NUMBER (include area code) 202-576-3551	d. TELEPHONE NUMBER (include area code) 202 576-3303
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Formal, S B
	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Kopecko, D J

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Immunochemistry; (U) Pilus protein; (U) Peptide Sequencing; (U) Synthetic Vaccine

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23(U) Development of peptides emcompassing the common binding domains of bacterial pili as immunizing agents against diarrheal and gonococcal infections. These two diseases are major health problems to military personnel.

24(U) Pili will be cleaved to yield peptides, which will be isolated, characterized, and assayed in binding studies. The Edman and nucleotide sequencing methods will be used to delineate the structure of the common domain peptides. Cloning of pili gene fragments into E. coli will be attempted to obtain additional structural information and to prepare synthetic vaccines.

25(U) 83 10-84 09 A large CNBr fragment of GC pilin has been sequenced up to 23 residues and shown to be derived from the amino-terminal end. The amino acid sequence, MePhe-T-L-G-E-M-I-V-I-A-I-V-G-I-A-A-V-A-L-L-P-A, indicate this fragment is hydrophobic and homologous with the N-terminal sequences of pili of Moraxella and Pseudomonas. Secondary structure prediction indicated the fragment existing predominantly in the beta sheet configuration.

An E. coli recombinant was found to express GC pilin protein by replicate blot assay. On SDS gels, the cloned protein migrated with similar mobility as native GC pilin; however, as evidenced by electron microscopy, it does not appear to be polymerized, thus suggesting incomplete processing.

A mixed DNA probe corresponding to the M-I-V-A-I sequence have been synthesized and will be used to isolate the cloned DNA fragment encoding GC pilus. (For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 to 30 Sept 84)

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 97: Immunochemistry on Binding Domain Peptides of Bacterial Pili

Investigators:

Principal: Robert C. Seid, Jr., Ph.D.

Associates: Samuel B. Formal, Ph.D.
Dennis J. Kopecko, Ph.D.
LTC Raymond C.Y. Chung, Ph.D.

Objectives

The objectives are (1) to delineate the "common" binding peptide domains of bacterial pilus proteins and (2) to develop these proteins as synthetic vaccines, either by themselves, or after conjugation in adjuvant carriers.

Progress

(1) A large purified CNBr fragment of Gonococcal P-3-2 pilin has been sequenced by the Edman degradation cycle up to 23 residues. This fragment was shown to be derived from the amino-terminal end due to an identification of an unusual amino acid N-methyl phenylalanine. The amino acid sequence established so far, MePhe-T-L-G-E-M-I-V-I-A-I-V-G-I-A-A-V-A-L-P-A, indicate that this fragment is hydrophobic. Moreover, the sequence is highly homologous with the N-terminal sequences of pili of Moraxella and Pseudomonas. Secondary structure prediction by the Chou-Fassman rules indicated that the sequenced hydrophobic fragment exists predominantly in the beta sheet configuration.

(2) An E. coli recombinant, carrying GC DNA fragment, was found to express GC pilin protein, by replicate nitrocellulose paper blot assay using a crossreactive monoclonal meningococcal pili antibody. On SDS gels, the cloned protein migrated with similar mobility as native GC pilin of the E. coli recombinant does not appear to be polymerized as native pili, thus suggesting incomplete processing or fold assembly of pilus subunits.

(3) A mixed oligonucleotide probe (15-mers), corresponding to the Met-Ile-Val-Ala-Ile sequence, have been synthesized on a solid support by the phosphoramidite method. The synthetic DNA oligomers, presently being analyzed by HPLC, will be used to isolate the cloned DNA encoding GC pilus.

(4) A chemical method was developed to prepare covalent conjugates between detoxified lipopolysaccharides and GC pili. Preliminary data from serological studies indicate that the antigenicity and immunogenicity of both gonococcal pili and lipopolysaccharides are enhanced as a result of covalent coupling.

Future Plans

Future objectives of this long term project are as outlined: Peptide fragments, generated by chemical and enzymatic cleavages of pilus protein and isolated by HPLC, will be sequenced by the Edman method, compared for homologies with other pili sequences, and tested in binding inhibition assays. Purification of the expressed GC pilus from *E. coli* bacteria will be attempted using biochemical techniques. The presence or absence of sugar moieties on pilus protein and their possible roles in binding will be investigated. The synthetic, mixed DNA oligomers will be used for hybridization studies and to deduce the nucleotide sequence of the portion of the GC DNA gene encoding the pilus protein. Peptides, 8 to 10 residues in length, encompassing both the conserved and variable regions, will be synthesized, covalently linked to immunopotentiators, and tested in laboratory animals.

Bibliography

1. Seid, R.C., Jr., H. Schneider, R. Nussbaum, H. Sidberry, and J.C. Sadoff. (1983). Antigenicity and immunogenicity of gonococcal pili-LPS conjugates. Abstract, 4th International Symposium on Pathogenic Neisseria, Asilomar Conference Center, Pacific Grove, CA.

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3. Zollinger, W.D., J.S. Ray, E.E. Moran, and R. Seid. (1984). Identification by monoclonal antibody of an antigen common to the pathogenic *Neisseria* species. Abstract, Fourth International Symposium of Pathogenic Neisseria, Asilomar Conference Center, Pacific Grove, CA.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 300522	2. DATE OF SUMMARY 84 09 30	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	H. Term	U	U		CX	

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	98WWJJ
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code) (U) Dynamic Regulation of Neurotransmitter Systems

12. SUBJECT AREAS
0616 Physiology 0510 Psychology 0615 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
82 10	84 10	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	80
85	0.0	0

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Neuropsychiatry
b. ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Mobley, W C
d. TELEPHONE NUMBER (include area code) 202 -576-3551	d. TELEPHONE NUMBER (include area code) 202 576-3028
21. GENERAL USE FINA MILITARY CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Holaday, J W g. NAME OF ASSOCIATE INVESTIGATOR (if available) Tortella, F C

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Lab Animals; (U) rats; (U) mice; (U) Nervous System; (U) Receptor; (U) Autoradiography; (U) Immunocytochemistry

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Although the existence of discrete cholinergic neurotransmitter systems is well known, still to be accomplished is a careful synthesis of physiogical, biochemical and morphological data which would indicate how these systems function normally, during development, in aged animals in response to chemical or electrolytic lesions. By employing sophisticated biochemical and morphological methodologies, we will investigate the baseline parameters for the cholinergic system and characterize the dynamic alterations which attend its operation under a variety of conditions. The effect of nerve growth factor and other neuropeptides on several parameters of cholinergic function will be tested in normal adults and under the conditions to be described. There is military relevance in this research.

24. (U) In the first series of experiments neonatal, adult and aged rats will receive intracerebroventricular injections of nerve growth factor (NGF). This protein will be prepared from the submaxillary glands of adult male mice. Injections will follow surgical preparation of animals by the aseptic placement of intracranial burr holes. Following injection, animals will be sacrificed at intervals for determination of choline acetyltransferase (ChAT) activity, high affinity acetylcholine uptake, autoradiographic confirmation of acetylcholine receptors and immunocytochemical staining fjor ChAT. In subsequent experiments, these same measures will be determined after exposure of normal rats to acetylcholinesteraseinhibitors and electroytic lesions of cholinergic pathways.

25 (U) 83-10-84-09. NGE has been purified to near homogenity via the use of a series of liquid chromatographic separations. This purified material retains its activity neurons in vivo. A robust rapid increase in cholineacetyltransterase (ChAT) activity is elicited in basal forehein neurons in neonatal rat. This increase is selective for (ChAT). A dose-response relationship has been demonstrated for NGF and ChAT in basal forebrain neurons. Immunaltochemical studies of ChAT positive neurites non human promates has indicated their presence in neuritic plaques. For technical report see Walter Reed Army Institute of Research annual Progress report 1 Oct 83-30 Sep 84.

Project : 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 98 Dynamic Regulation of Neurotransmitter Systems

Investigators:
Principal: Mobley, W.C., M.D., Ph.D.
Associate: Holaday, J.W., PH.D.; Tortella, F.C., Ph.D.

Objectives:

Problems investigated were: (1) What is the normal anatomy of the cholinergic cells of the basal forebrain? (2) What biochemical parameter characterize these cells? The studies include determination of the specific neurotransmitter enzyme sholine acetyltransferase (ChAT), high affinity choline uptake, and acetycholine receptor analysis; (3) What anatomical and biochemical features describe this system during normal development and normal agine? (4) What changes result from administration of a potential trophic factor, nerve growth factor (NGF)? What is the role of other neuropeptides in these changes? and (5) What changes occur after intoxication with anticholinesterase agents and electrolytic lesions, and do such treatments alter the response of cholinergic neurons to NGF?

Approach:

These included: (1) Anatomical studies will employ immunocytochemical techniques with a monoclonal antibody directed against ChAT. Cholinergic cell bodies and their fibers will be mapped; (2) Enzyme levels of ChAT and acetalcholinesterase will be determined in the brain. Standard procedures will be uksed to measure choline uptake. Standard procedures will be used to measure choline uptake. Acetylcholine receptor density will be determined autoradiographically; (3) Purified NGF will be injected intracerebroventricularly and, in subsequent experiments, directly into specific nuclear groups. Determinations will be made of NGF receptors and of specific NGF retrograde flow; and (4) acetylcholinesterase inhibitors will be administered intravenously.

Progress:

Immunocytochemical studies with monoclonal anti-ChAT antibody have revealed quite specific nuclear groups of cholinergic cells. Anatomical studies of neonatal and aged animals are in progress. We have identified bizarrely shaped, twissted neurites in the neocortex of aged non-human primates and have found that some such profiles are present in neuritic plaques. The latter finding indicates that cholinergic neurites participate in plaque formation and indicates that these neurites may be the morphological correlate of distributed cholinergic function in the memory disorder of Alzheimers disease. Neonates receiving an intraventricular injection of NGF demonstrate dramatic increases of ChAT activity inbasal forebrain nuclei. These increases are specific to ChAT. Highly purified NGF samples evoke these same responses. A dose-response relationship for NGF and ChAT activity has been determined. This relationship indicates that nuclear and fiber projection areas respond differently. NGF's effect on ChAT are apparent as early as 2 days following injectin.

This project has provided strong morphological evidence for involvement of cholinergic systems in memory disorders and suggests that similar morphological correlates may attend intoxication or other lesions of cholinergic neurons. It has also demonstrated that a purified trophic factor strongly influences a key biochemical parameter of cholinergic neurons and suggests that it may also influence the viability of these neurons and their level of function. If true, this would suggest that NGF may be capable of improving or restoring cholinergic function to traumatized or intoxicated animals and humans.

For additional information see 1984 Annual Report, Project 3E162777A879.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 300525	84 09 30	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	H. Term	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	99 WWN6
b. CONTRIBUTING				
c. CONTRIBUTING	NONE			

11. TITLE (Precede with Security Classification Code)
(U) Pathogenesis of Campylobacter jejuni in Laboratory Animals

12. SUBJECT AREAS
0613 Microbiology 0603 Biology 0606 Environmental Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
82 10	84 10	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER				
c. TYPE	d. AMOUNT	84	1.0	20
e. KIND OF AWARD	f. CUM/TOTAL	85	0.0	0

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS Division of Veterinary Medicine Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Bartz, C R
d. TELEPHONE NUMBER (include area code) 202 576-3551	d. TELEPHONE NUMBER (include area code) 202 576-3019/2071
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Brendle, J J g. NAME OF ASSOCIATE INVESTIGATOR (if available) Sims, R E

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Campylobacter fetus subsp jejuni; (U) Pathogenesis
(U) Antibiotic Resistance; (U) Plasmids; (U) Monoclonal Antibody; (U) Lab Animals;
(U) Volunteers; (U) Dogs; (U) Wolves; (U) Aotus; (U) Baboons; (U) Mice; (U) Rabbits; (U) Qua

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The technical objectives of this work unit are to determine whether passage of Campylobacter jejuni isolates can be made under experimental conditions and if passage is successful, to determine any pathogenesis in the animal model obtained. Isolates from humans, bush dogs, maned wolves, aotus, baboons, and domestic dogs will be investigated. C. jejuni occurs in a large number of species from all areas of the world and has been implicated in disabling gastroenteritis which has been noted as an obstacle to fielding of the rapid deployment force.

24. (U) Isolates will be characterized by antibiotic sensitivity, plasmid presence, and serology prior to infecting laboratory animals. Infectivity and pathology will be correlated with said characteristics. Monoclonal antibodies will be developed against cell wall portions to aid in understanding the bimorphism (spiral and cocci) of the organism at different stages of the growth cycle and as a means of determining significant antigenic portions of the cell surface.

25. (U) 8310-8409 This work unit has been terminated. Campylobacter jejuni isolates from a wide variety of human, laboratory animal and zoo animal sources have been characterized by antibiotic resistance and plasmid profiles. A collection of 265 isolates has been preserved for future studies. Procedures and media have been developed for the isolation, growth and characterization of these organisms and a methodology developed for routine and reliable plasmid extraction. No animal model for Campylobacteriosis could be developed; however, the nude mouse and the Coturnix quail would be useful for certain studies of pathogenic mechanisms. For technical report see WRAIR Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

PROJECT: 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

WORK UNIT: 99 Pathogenesis of *Campylobacter jejuni* in Laboratory Animals

INVESTIGATORS:
Principal: Curtis R. Bartz, MAJ, VC
Associates: James J. Brendle, DAC; Robert E. Sims, DAC
Assistants: SP5 Germaine Flores; SP4 Naomi Capozza

OBJECTIVES:

The objectives of this work unit were to:

a. Collect fundamental data on *Campylobacter jejuni*
b. Determine the suitability of different animals as models of Campylobacteriosis in humans

IMPORTANCE:

Basic information is required on animal reservoirs, antibiotic resistance patterns and plasmid content of *C. jejuni* for empirical treatment regimes and prophylaxis. An animal model of disease is required for study of the pathogenesis of *C. jejuni* enteritis, of which little is known.

PROGRESS:

Since October 1983, the screening of laboratory animals for *C. jejuni* continued. 130 isolates were added to the *C. jejuni* culture collection and data base. The majority of the isolations were from newly arriving dogs. During the year, from 30 to 50% of the dogs in each group of arrivals harbored *C. jejuni*.

The addition of IsoVitaleX to culture media enhanced the growth of *C. jejuni* on agar and in broth. This in addition to slight modifications of the plasmid extraction protocol resulted in increased yields on a more consistant isolation of individual plasmids.

Preliminary experiments on selected isolates for the detection of bacteriocins by the agar overlay method and for toxin production by tissue culture methods gave promising results. These protocols will be further refined to determine whether or not bacteriocins or toxins are actually present.

Selected isolates were supplied to the Division of Biochemistry for in depth studies of antibiotic resistance plasmids, a search for bacteriophage and development of protocols for conjugation and transformation experiments for further genetic studies.

This work unit will be terminated. The diagnostic aspects of the project will be integrated into the routine of the diagnostic laboratory of the Division of Veterinary Medicine. The more promising avenues for research uncovered by this investigation have been integrated into ongoing projects in the Division of Biochemistry.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 300527
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	100 WWP8
b. CONTRIBUTING				
c. CONTRIBUTING	NONE			

11. TITLE (Precede with Security Classification Code)
(U) Oral Vaccination of Peyer's Patches and Mucosal Surfaces

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE: 82 10
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-house

17. CONTRACT GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	1.0	100
85	1.0	100

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
Top, F H JR
d. TELEPHONE NUMBER (include area code)
202 576-3551

20. PERFORMING ORGANIZATION
a. NAME Division of Pathology
Walter Reed Army Institute of Research
b. ADDRESS
Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
Roy, M J
d. TELEPHONE NUMBER (include area code)
202 -576-3053
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
Tseng, J
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
Alving, C

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Microfold cells; (U) Peyer's patches; (U) Mucosal Surfaces; (U) Oral immunization; (U) Gastrointestinal immunity; (U) Secretory immunity; (U) lab animals; (U) rabbits

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23 (U) The primary objectives are to study the processes of antigen sampling, attachment, uptake and presentation within the folicle-associated epithelium (FAE) of Peyer's patches in th small intestine. This will be accomplished by studying the derivation and characteristics of the cells involved and the dynamics of antigen trapping and processing in FAE. Knowledge derived from these studies will be used to manipulate the mucosal immune system by enhancing ant gen attachment and presentation, thereby promoting oral immunization while preventing establishment of oral tolerance. The military relevance of this research is prevention of respiratory and gastrointestinal infections.

24 (U) Cell surface receptors on FAE microfold (M) cells will be characterized on the basis of lectin, immunoglobulin and antigen binding capabilities. Leukocytes of the FAE, grouped o the basal surface of M cells, will be classified as T cells, B cells, macrophages or a combination of these cell types, on the basis of well defined surface and cytoplasmic markers. Possible binding of oral antigens by FAE leukocytes will be determined, also. Immunocytochemistry and autoradiography will be used as basic techniques.

25 (U) 83 10 - 84 09. Monoclonal antibodies have been prepared against FAE cells and intestinal macrophages. UEA, a lectin that preferentially binds the FAE, was used to enrich appendicular FAE cells. Monoclonal antibodies or UEA, entrapped in liposomes along with microbial antigens, will be a means of delivering the antigens to gut associated lymphoid tissues and thereby selectively immunizing mucosal surfaces. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 October 1983 - 30 September 1984.

EDITION OF MAR 68 IS OBSOLETE.

PROJECT 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 100: Oral Vaccination of Peyer's Patches and Mucosal Surfaces

Investigators:
Principal: Michael J. Roy, Ph.D
Associates: Jeenan Tseng, Ph.D
Carl Alving, COL, MC

Problem:

More effective methods are needed to selectively deliver antigens to cells involved in the afferent arm of secretory immune responses.

Importance:

Infections of mucosal surfaces, particularly the gastrointestinal, respiratory and genital tracts, result in a substantial loss of military manpower. Effective oral vaccines have not been developed for most pathogens that traverse or infect mucosal tissues, in large part because traditional methods of immunization result in poor secretory immune responses.

Objectives and Approach:

Our primary objective is to develop safe, effective means of delivering microbial antigens to gut-associated lymphoid tissues. To achieve this goal, we wish to determine which antigen receptors are unique to lymphoepithelial cells, particularly M cells.

The epithelium of gut associated lymphoid tissues consists of three cell types: M cells, columnar enterocytes and lymphocytes. M cells are actively involved in the uptake of antigens and the subsequent transport of these materials to underlying lymphoid tissues. It is hypothesized that these events are crucial for effective oral immunization to occur. Since M cell surface molecules are likely involved in antigen binding and transport, the lymphoepithelium will be studied in more detail using three experimental approaches: 1) searching for lectins or neoglycoproteins that bind exclusively to M cells; 2) isolating and culturing M cells so that antigen binding capabilities can be studied in vitro; 3) producing monoclonal antibodies reactive with M cell surface glycoproteins involved in antigen binding and uptake; 4) subsequently delivering antigens to the lymphoepithelial cells by feeding carriers, such as liposomes, into which both the ligand and a toxin or an antigen has been incorporated.

Progress:

Previous morphologic studies on adult rabbit appendix, Peyer's patches and sacculus rotundus show these tissues to be a rich source of M cells. Rabbit M cells possess no alkaline phosphatase, some lysosomal acid phosphatase and significant nonspecific esterase activity. These cytochemical characteristics are extremely helpful in identification of M cells *in situ* or *in vitro* and they can be used as enzymatic profiles when examining the nature and development of M cells. These enzymes are, however, primarily within the M cell cytoplasm and therefore they are of limited value in defining antigen receptors or in cell isolation and enrichment.

Morphologic and histochemical studies of developing neonatal rabbit lymphoepithelium have established that T lymphocytes, but not M cells, are present at birth. The M cells are morphologically distinguishable and always in contact with T lymphocytes by the second day of life. The enyzmes nonspecific esterase and acid phosphatase develop during the second week of life in neonatal M cells.

Previously it had been demonstrated that the lectin *Ulex europaeus* agglutinin I (UEA) bound to adult appendicular lymphoepithelial cells, but not to villar epithelial cells. In neonatal rabbits, the UEA-binding glycoconjugates developed in lymphoepithelial cells on the second day of life and for the next two weeks these molecules were a unique property of cells covering gut associated lymphoid tissues.

Lymphoepithelial cells, including M cells, were, for the first time, selectively dissociated from GALT. The rabbit lymphoepithelial cells were characterized as regards ultrastructural morphology, enzymes (histochemistry) and UEA-Binding glycoconjugates. Mice were immunized with these lymphoepithelial cells, hybidomas prepared from the splenocytes and several clones, each producing monoclonal antibody to gut-associated lymphoid tissue molecules, were isolated. Two clones, of particular interest were WR25L10, specific for an antigen on GALT macrophages including those directly beneath the lymphoepithelium, and WR30E5 which binds to a molecule that circumscribes the nests of lymphoepithelial lymphocytes.

Experiments aimed at using liposomes as carriers of antigen or ligands have begun. Liposomes with UEA have been introduced into the rabbit small intestine and appendix. Preferential binding of the liposome - UEA complexes to the lymphoepithelial surface will be established by electron microscopy.

Future Objectives:

We will continue attempting to enrich M cells in lymphoepithelial cell populations and producing monoclonal antibodies to lymphoepithelial cells under the current protocol; the ultimate goal is to obtain an antibody that is specific for M cell surface receptors. We will inject liposomes, containing UEA, WR25L0, WR30E5, or other promising monoclonals into the rabbit gut and determine if; 1) there is preferential binding of the liposome-ligand to the lymphoepithelial surface; 2). There is uptake of liposome-ligands by the lymphoepithelium; 3) liposome-ligands eventually reach follicles of GALT; and 4) liposome ligand-antigen complexes, when fed to rabbits, induce specific secretory immune responses. Achievement of these goals in the rabbit could be repeated using human lymphoepithelial cells from Peyer's patches. Eventually, we hope to develop a method for presenting a variety of antigens to mucosal surfaces in a way that stimulates strong, long lasting secretory immunity to infectious diseases, with potential direct application to vaccine development and efficacy.

Formal Presentations:

Roy, M.J. 1983. Preferential binding of *Ulex europaeus* agglutinin (UEA) to nonlymphoid cells in the appendix and mesenteric lymph nodes of adult rabbits - American Society of Biological Chemists, St. Louis, MO, 10 Jan 1984; *Federation Proceedings*

Roy, M.J. 1983. Development of the Lymphoepithelium in neonatal rabbit appendix; Morphologic and lectin-binding studies. American Gastroenterological Association, New Orleans, LA, 19 May 1984; *Gastroenterology* 86:1224. (1984)

RESEARCH AND TECHNOLOGY WORK T SUMMARY	1. AGENCY ACCESSION DA 300531	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636	

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	101 WWQ8
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code) (U) Effects of Thai Medicinal Plant Preparations on in vitro Development of Plasmodium falciparum

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE 82 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	0.5	50
85	0.5	55

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER (include area code)
202 576-3551

20. PERFORMING ORGANIZATION
a. NAME
AFRIMS
b. ADDRESS
Bangkok, Thailand
c. NAME OF PRINCIPAL INVESTIGATOR
WEBSTER, H
d. TELEPHONE NUMBER (include area code)
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
PAVANAND, K.
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE
FINA
MILITARY CIVILIAN APPLICATION H

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Malaria; (U) Plasmodia; (U) Medicinal plants; (U) Infectious Diseases

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The technical objective is to determine whether selected Thai medicinal plants and their chemically isolated components exhibit an inhibitory effect on the in vitro growth of P. falciparum. There is a military requirement for new therapeutic agents to prevent and treat malaria.

24. (U) The approach involves chemical preparation of crude plant extracts for in vitro screening followed by isolation and purification of components for in vitro antimalarial activity confirmation, structure determination and mechanism of action studies.

25. (U) 83 10 - 84 09. During this period the investigators isolated and characterized a compound from a Thai medicinal plant (Brucea javanica L. Merr). This compound which was designated Brucine A, was submitted to Walter Reed Army Institute of Research Division of Experimental Therapeutics for in vitro evaluation. The assays were performed with two cloned Plasmodium falciparum isolates, Sierra Leone D-6 and Indochina W-2 using chloroquine, melfoquine, quinine, ginghoosu, and halofantrin on the same microtites plates as controls. These tests indicated that Brucine A was as effective as chloroquine and mefloquine and more effective than quinine when tested against the Sierra Leone D-6 isolate and more effective than chloroquine and quinine when tested against the Indochina W-2 isolate. In related studies it was shown that crude preparation of Brucine C (a related compound from the same source as Brucine A) is more active than Brucine A. Current efforts are concentrated on fu ther purification of Brucine C. For technical report see Walter Reed Army Institute of Research Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

PROJECT: 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 101: Effects of Thai Medicinal Plant Preparation on
In vitro Development of *Plasmodium falciparum*

Principal Investigator: MAJ H. K. Webster, MSc

Thai Medicinal Plants as a Source for New Leads in the Design of Antimalarial Compounds

PROBLEM: The continued worsening problem of antimalarial drug resistance coupled with our present ignorance about the parasites basic biochmistry requires that we consider novel alternatives for the identification and design of new antimalarial drugs. Herbal remedies for malaria, identified from traditional medicine sources, offer important leads for the discovery of compounds with antimalarial activity.

PROGRESS: A number of medicinal plants with putative antimalarial properties have ben identified from Thai traditional medicine records. To date, herbal preparations from six plant families have been screened in our laboratory for antimalarial activity against multi-drug resistant *P. falciparum.* The plant families were: *Simaroubaceae, Plumbaginaceae, Apocynaceae, Cleomaceae, Moraceae* and *Bignoniaceae.* One plant species, *Brucea javanica* (L.) Merr, of the *Simaroubaceae* has proven most promising. Primary screening using morphological techniques revealed the crude chloroform extract of the fruit of *Brucea javanica* to have the most potent antimalarial activity. Three different active compounds were isolated and purified from the plant's fruit using combined column and preparative thin layer chromatographic techniques. Structural determination was made by mass spectroscopic, IR, NMR and single crystal X-ray analysis. The three compounds were shown to be quassinoids: bruceine A, bruceine B hydrate and bruceine C. Confirmation of the three compounds antimalarial activity was done *in vitro* using a radioisotope microdilution technique. The antimalarial activity of bruceine A and bruceine B hydrate was comparable to that of mefloquine (ID 50's of 8.66, 8.15 and 6.26 ng/ml respectively). Bruceine C was, however, more active than mefloquine (ID 50 of 1.95 ng/ml). All three bruceine compounds were observed to be effective *in vitro* against a mefloquine resistant isolate of *P. falciparum.*

RECOMMENDATION: The isolation and elucidation of the chemical structure of different active components from the medicinal plant, *Brucea javanica* is of major value in that it identifies new lead compounds for synthesis of potential antimalarial drugs. Such alterative sources of lead compounds are much needed for the continued struggle against multi-drug resistant falciparum malaria. This work should be expanded to other medicinal plants and definitely continued.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 300532
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	102 WWI9
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code): (U) Pharmacokinetics of Atropine and L-hyoscyamine

12. SUBJECT AREAS: 0615 Pharmacology 0616 Physiology

13. START DATE: 82 12
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-house

17. CONTRACT/GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	1.5	33
85	1.5	28

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code): Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL: [illegible], F h JR
d. TELEPHONE NUMBER (include area code): 202 576-3551

20. PERFORMING ORGANIZATION
a. NAME: Division of Medicine
b. ADDRESS:
Walter Reed Army Institute of Research
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR: SMALLRIDGE, R C
d. TELEPHONE NUMBER (include area code): 202 5763014
f. NAME OF ASSOCIATE INVESTIGATOR (if available): WHORTON, N E
g. NAME OF ASSOCIATE INVESTIGATOR (if available): FEIN, H G

21. GENERAL USE: FINA
MILITARY CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Pharmacokinetics; (U) Atropine; (U) Drug Metabolism; (U) ~~Human~~ Volunteers; (U) Rabbits (U) Lab Animals;

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23.(U) To develop a radioimmunoassay for atropine. To determine the pharmacokinetics and tissue distribution of atropine. To determine the pharmacokinetics in humans. Atropine is the single most important drug used to treat casualties from exposure to nerve agents. There is a paucity of information on drug metabolism of atropine in normal subjects, and no information on its fate under conditions known to alter an individual's physiologic response to the drug.

24.(U) A sensitive and specific radioimmunoassay for atropine will be used. After either intramuscular or intravenous administration, drug metabolism will be determined in animals and humans by measuring drug concentrations for kinetic analysis. Tissue concentrations will be measured in animals, particularly with respect to the degree to which atropine sulfate penetrates the blood-brain barrier.

25.(U) 8310-8409. The atropine antibody was further characterized and its utility validated. Support was provided for a civilian contract for measuring human serum atropine blood levels after inhalation of the drug. A protocol was approved for studying atropine pharmacokinetics and the psychophysiologic responses to varying drug doses in normal human volunteers. Two animal protocols were prepared and approved for studying (a) The effects of hypovolemia, (b) route of administraion, and (c)the effect of metabolic alterations on atropine metabolism (in progress). For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 to 30 Sep 84.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

Project: 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit: 102 Pharmacokinetics of Atropine and L-hyoscyamine

Investigators:
  Principal: Robert C. Smallridge, LTC MC
  Associate: Nancy E. Whorton, GS-11
  Carolyn Umstott, GS-7
  Henry Fein, MAJ MC

Atropine is the first-line drug used to treat combat casualties from exposure to chemical warfare nerve agents, and it is issued to soldiers in auto-injectors. There is considerable information about the physiologic responses to selected doses of atropine (1-4), and it is recognized that a variety of independent variables may alter one's response to a fixed dose (e.g., extremes of climate, exercise, co-administration of other drugs, protective garments). However, there is scant information on the absorption, distribution, and elimination of atropine in normal individuals (5-7), and no studies of drug metabolism in situations which might be affecting drug metabolism. It is quite possible that the reason why a specific dose of atropine is well tolerated under certain conditions, yet can physically incapacitate a soldier under other circumstances, is due to alterations in the metabolism of the drug. To assess this possibility, and, thence, to determine the appropriate dose for treatment under given conditions, knowledge of the pharmacokinetics of the drug is required. This information is currently unavailable. The objectives of this Work Unit are to: (1) develop radioimmunoassays to measure small quantities of atropine and L-hyoscyamine (the active component in atropine) in blood, urine, and tissues; and (2) determine the rates of absorption and elimination of atropine from the body, based on serial blood levels of atropine after administration.

This was a new Work Unit in FY83. Progress to date has been primarily the development of a highly sensitive radioimmunoassay for atropine. The atropine antibody was produced in the following manner. Atropine was conjugated to bovine serum albumin by initially conjugating the diazotized p-amino-benzoic acid to atropine and then forming an amide linkage between the amine groups of the protein and the carboxyl group of benzoic acid. Four male New Zealand white rabbits were immunized with this atropine-BSA immunogen. The rabbits were first bled two months after the initial immunization procedure and antibodies were detectable at that time. To obtain optimum sensitivity, the antibodies generated were used in the assay at a final dilution of 1:9000. Pre-immunization serum failed to bind any tritiated atropine. The standard curve is linear up to 2 ng of atropine. Identical standard curves were obtained using human plasma, serum, urine, or phosphate-buffered saline, pH 7.5, indicating the lack of interfering substances in these body fluids. The assay reliably detects as little as 15 pg of atropine, and a 50% inhibition of binding of the ^{3}H-atropine ligand to the antibody is attained with 140 pg of atropine. The antibody recognizes both L- and D- forms of the drug, the L- form being slightly preferable. The atropine hydrolysis products, tropine and tropic acid, do not cross-react with the antibody. The interassay and intraassay coefficients of variation are 13.5% and 8.3%, respectively, and recoveries range from 94-102%. Excellent displacement curves and recoveries have been documented in a variety of species, including man, monkey, dog, cat, sheep, goat, rabbit, rat, guinea pig, and chicken. Thus, the assay is adaptable for use in a number of animal models.

A human use protocol for determination of atropine pharmacokinetics in normal men is near completion, as are two animal protocols. All three studies should be conducted in FY84.

Recommendations for the future first involves an assessment of atropine and L-hyoscyamine pharmacokinetics in normal situations. This should be followed by similar studies under conditions known to alter the dynamic responses to a fixed dose of atropine. From these studies should emerge guidelines for optimum drug dosages under a variety of situations, based upon pharmacokinetic principles.

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Formal Presentations

1. Verma, P.S., R.G. Adams, and R.L. Miller, Reactivity of plasma kallikrein-kinin system during sickle cell crisis. In International Congress on Pediatric Lab Med Toronto, Canada, May 29-June 2, 1983.

2. Verma, P.S., R.F. Hoyt, Jr., A.J. Jackson and Y.Y. Phillips, Radioimmunoassay (RIA) for desmosine and study of its pharmacokinetics. Fed Proc 42:1298, 1983. Presented at the Federation of American Societies for Experimental Biology, Chicago, Il.

3. Verma, P.S., D.E. Butkus, and R.G. Adams, Inhibition of rat kidney angiotensin converting enzyme by arginine vasopressin Fed Proc 42:1948, 1983. Presented at the American Society of Biological Chemists, San Francisco, CA

4. Verma, P.S., R.G. Adams, and R.L. Miller, Activation of plasma kallikrein-kinin system in patients during sickle cell crisis. Clin. Chem 29:1266, 1983. Presented at the American Society of Clinical Chemists, New York City, NY.

Bibliography

1. Verma, P.S., C. Umstott, and R.C. Smallridge, Development of an atropine radioimmunoassay suitable for use in a variety of animal models (submitted for presentation).

2. Adams, R.G., P.S. Verma, A.J. Jackson, and R.L. Miller, Plasma pharmacokinetics of intravenously administered atropine in normal human subjects. J Clin Pharmacol 22:477, 1982.

3. Miller, R.L., P.S. Verma, and R.G. Adams, Studies of the kallikrein-kinin system in patients with sickle cell anemia. J Nat Med Assoc 75:551, 1983.

4. Verma, P.S., R.G. Adams, and R.L. Miller, Reduced plasma kininogen concentration during sickle cell crisis. Res Comm Chem Pathol and Pharmacol 41:313, 1983.

5. Verma, P.S., R.F. Hoyt, Jr., A.J. Jackson, and Y.Y. Phillips, Pharmacokinetics of intravenously administered desmosine in sheep. Conn Tissue Res (In Press).

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA 300763	2. DATE OF SUMMARY 84 10 01	*REPORT CONTROL SYMBOL* *DD-DR&E(AR) 636*
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	103 WWHB
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE *(Precede with Security Classification Code)*
(U) Membrane Fusion and Diffusion of Receptors in Cells

12. SUBJECT AREAS
0601 Biochemistry 0603 Biology 0615 Pharmacology

13. START DATE 83 01	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-house

17. CONTRACT GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		84	0.5	25
c. TYPE	d. AMOUNT	85	0.5	28
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Biochemistry
b. ADDRESS *(include zip code)* Washington, DC 20307-5100	b. ADDRESS Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Chiang, P K
d. TELEPHONE NUMBER *(include area code)* (202)-576-3551	d. TELEPHONE NUMBER *(include area code)* (202)-576-1361
21. GENERAL USE FINA	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Doctor, B P
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Gordon, R K

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Receptors; (U) Enzymes; (U) Membranes; (U) Lipid Bilayers

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The object of this work unit is to study the fusion and diffusion of receptors of cells reconstituted into phospholipid bilayers introduced to other cell types as studied biochemically or by fluorescence microscopy. There is military relevance in this research.

24. (U) Research focuses on the characterization and isolation of receptors of cells. The receptor studies are mainly muscarinic and nicotinic receptors of the following cell lines; N4TG1 neuroblastoma and NG108-15 neuroblastoma x glioma hybrid cells. After initial characterization of the receptors in the cell lines, their isolation will be carried out from either the cell lines or brain tissues. The ultimate goal is to purify and reconstitute the receptors, and to introduce the latter into phospholipid bilayers. For example, the fusion of muscarinic receptors into a noncholinergic cell allows the testing of the muscarinic functions in that cell. Whether the cell with new receptors inserted can exhibit muscarinic functions will be tested by radioactive ligand binding (such as [^{3}H]QNB), cyclic GMP coupling effect, and phosphatidyl inositol turnover. Fluorescence microscopy will also be used to monitor the diffusion and translocation of receptors into cells.

25. (U) 83 10 - 84 09: Affinity labeling agents for the muscarinic receptors have been synthesized. Procedures are being developed to couple them to Sephadex columns to be used in affinity chromatography for the purification of the receptors. Moreover, a new class of antimuscarinic agents have been discovered. They are structural analogs of S-isobutylthio-adenosine. These analogs inhibit the binding of [^{3}H]quinuclidinyl benzilate to the muscarinic receptors of N4TG1 neuroblastoma cells, and also inhibit the contraction of guinea pig ileum stimulated by acetylcholine.

DD FORM 1498, 83 MAR — EDITION OF MAR 68 IS OBSOLETE.

PROJECT: 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

WORK UNIT: 103 Membrane Fusion and Diffusion of Receptors in Cells

INVESTIGATORS:

Principal:	Peter K. Chiang, Ph.D.
Associate:	Richard K. Gordon, Ph.D.; B.P. Doctor, Ph.D., SP6 George A. Miura, Ph.D., Jarle Aarbakke, M.D., Ph.D.
Assistant:	SP4 Felipe N. Padilla, Michelle M. Richard

The objective of this work unit is to study, characterize and isolate receptors of cells. The ultimate goal is to re-constitute the isolated receptors into lipid bilayers and to introduce them to cell types lacing those receptors and to follow the fusion and diffusion of the receptors in the host cells. The following investigations were conducted:

1. Aldosterone stimulates methylation reactions and membrane events in cultured toad bladder epithelial cells:

The effect of aldosterone (Aldo) on phospholipid (PL) biosynthesis in cultured toad bladder epithelial cells was studied in cells incubated with [1,2-^{14}C]choline and [methyl-^{3}H]methionine over a 5 hour period. Aldo (10^{-7} M) did not alter the uptake of either precursor but significantly stimulated the incorporation of both labels into phosphatidyl-choline (PC), the only PL labeled. ^{3}H-labeling of PC increased 29% and ^{14}C incorporation into PC increased 34% in cells exposed to Aldo. A similar 30% increase in protein carboxymethylation occurred in cells treated with Aldo. 3-Deazaadenosine (dzAdo), a methylation inhibitor, abolished the Aldo-stimulated increase in PC labeling from [^{3}H]-methionine. PC labeling from [1,2-^{14}C]choline was not affected by dzAdo. Basal and Aldo-stimulated protein carboxymethylation were inhibited by dzAdo. dzAdo (300)M) caused a mild decrease in basal short circuit current (I_{sc}) but completely inhibited the I_{sc} response to 10^{-7} M Aldo. Inhibition was complete when DZA was added up to 2 hrs following exposure to Aldo, and was reversible. Cells previously exposed to Aldo showed a significant increase in I_{sc} within 2 hrs following removal of dzAdo. We concluded that Aldo stimulates PL-methylation, protein carboxymethylation and turnover of PC from choline. Inhibition of methylation reactions coincides with the inhibition of I_{sc} response to Aldo.

2. Aldosterone Stimulated Transmethylations Are Linked to Sodium Transport

The effect of aldosterone (Aldo) on phospholipid (PL) biosynthesis in cultured toad bladder epithelial cells was studied in cells incubated with [1,2-^{14}C] choline and [methyl-^{3}H] methionine over a 5-hour period. Aldo (10^{-7}M) did not alter the uptake of either precursor but significantly stimulated the incorporation of both labels into phosphatidylcholine (PC), the only PL labeled. ^{3}H-labeling of PC increased 29% and ^{14}C incorporation into PC increased 34% in cells exposed to Aldo. A similar 30% increase in protein carboxymethylation occurred in cells treated with Aldo. 3-Deazaadenosine (dzAdo), a methylation inhibitor, abolished the Aldo-stimulated increase in PC labeling from [^{3}H] methionine. PC labeling from [1,2-^{14}C] choline was not circuit current (I_{sc}) but completely inhibited the I_{sc} response to 10^{-7} M Aldo. Inhibition was complete when dzAdo was added up to two hours following exposure to Aldo, and was reversible. Cells previously exposed to Aldo showed a significant increase in I_{sc} within two hours following removal of dzAdo. We conclude that Aldo stimulated PL-methylation, protein carboxymethylation and turnover of PC from choline. Inhibition of methylation reactions coincides with the inhibition of I_{sc} response to Aldo.

3. Methylation Increases Sodium Transport into A6 Apical Membrane Vesicles: Possible Mode of Aldosterone Action

When isolated apical membrane vesicles prepared from cultured A6 epithelia were incubated in vitro with the methyl donor S-adenosylmethionine, the control rate of amiloride-inhibitalbe sodium transport was doubled. The methylation inhibitors 3-deazaadenosine and S-adenosyl homocysteine returned the S-adenosylmethionine-stimulated sodium transport to control levels. Neither these agents nor adenosine affected sodium transport into control vesicles. In vesicles prepared from A6 cells treated with aldosterone, sodium transport was twice the control value and S-adenosylmethionine did not cause any further stimulation of transport. In those vesicles, both lipid and protein methylation were increased. These results suggest that methylation, which increases the rate of amiloride-sensitive sodium transport, is involved in the action of aldosterone at the apical membrane level in epithelia.

PUBLICATIONS

Sariban-Sohraby, S., Burg, M., Wiesmann, W. P., Chiang, P. K., and Johnson, J. P.: Methylation increases sodium transport into A6 apical membrane vesicles: a possible mechanism for aldosterone action. Science, 225, 745-746 (1984).

Wiesmann, W. P., Johnson, J. P., Miura, G. A., and Chiang, P. K.: Aldosterone stimulated transmethylations are linked to sodium transport. Am. J. Physiol., in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 300022	84 09 30	DD-DR&E(AR) 636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 [illegible]	H. Terminatioo	U	U		CX	

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	CC	104 WWH8
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) Development of Monoclonal Antibody - Producing Hybridomas

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	84 10	DA	C. In-house

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	1.0	85
c. TYPE	d. AMOUNT	85	0.0	0
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Biochemistry
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Gemski, P
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-2594
21. GENERAL USE FINA	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Gentry, M K
MILITARY/CIVILIAN APPLICATION H	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Lab Animals; (U) Mice; (U) Hybridoma; (U) Monoclonal Antobody; (U) Virus; (U) Toxin

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The technical objective is to produce hybrid cell lines secreting monoclonal antibodies using cell fusion techniques in a mouse-mouse hybridization system. Antigens include viruses, bacterial LPS antigens, toxins, enzymes, macrophage suppressor factors, and peptides. There is military relevance in this research.

24. (U) Spleen cells will be exposed to antigens and subsequently fused with mouse plasmacytoma cells. Fused cell products will be screened for production of antibodies and selected for cloning and further characterization on the basis of their reactivity in serological and biochemical tests.

25. U) 83 10 - 84 09: Monoclonal antibodies against organophosphate-coupled acetylcholinesterase (Torpedo fish) were produced; 7 antibodies preferentially bind to organophosphate enzyme rather than the non-poisoned form. Other antibodies are suitable for in situ localization of enzyme in tissues. Antibodies against human butyrylcholinesterase have been isolated; 2 antibodies appear to be directed to sites in proximity to the active site. Purified A and B toxins of C. difficile have been used to produce monoclonal antibodies; all antibodies obtained react with both toxins, although ratios of binding vary from less than 1 to greater than 5. A60 antigern from BCG, encapsulated in liposomes, was used to immunize animals. Some antibodies exhibit specificity for A60 vs. M. microti antigen. Polyclonal antibody has been produced in hyperimmune ascitic fluid against a synthetic nonapeptide fragment of cAMP-dependent protein kinase. Acetylcholinesterase from fetal bovine serum as an immunogen has produced antibodies recognizing that enzyme, reduced and denatured enzyme, and/or enzyme from Torpedo source. For technical report see Walter Reed Army Institute of Research Progress Report, 1 Oct 83 - 30 Sep 84. Work unit is being terminated by expiration of three-year funding period. This is a final report.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

PROJECT: 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

WORK UNIT: 104 Development of Monoclonal Antibody-Producing Hybridomas

INVESTIGATORS:

Principle:	Peter Gemski, Ph.D.
Associates:	Mary K. Gentry, B.S.; SP4 Karla K. Kopec, B.S.
In collaboration with:	MAJ J.E. Williams, MSC (DCD&I); Scott B. Halstead, M.D.; CPT E.A. Henchal, Ph.D., MSC (DCD&I); Walter E. Brandt, Ph.D. (DCD&I); David M. Morens, M.D.; Joel M. Dalrymple, Ph.D. (USAMRIID)

DESCRIPTION:

The development and isolation of antibody-secreting hybridoma cell lines to antigens of military interest and importance makes possible the production of unlimited supplies of monoclonal antibodies to these antigens for diagnostic work, vaccine development, epidemological studies, molecular cloning, and antigen mapping.

A. Monoclonal Antibodies to Plague (F1) Antigen.

1. Use of an Enzyme-linked Immunosorbent Assay to Measure Antigenaemia during Acute Plague.

An enzyme-linked immunosorbent assay (ELISA) was developed to measure concentrations of the specific F1 antigen of the plague bacillus in biological fluids. The assay employed a monoclonal antibody to capture the antigen. Sensitivity of the assay was 0.4 ng of F1 antigen. ELISA-inhibition was used to confirm the sensitivity of the reactions. This assay detected F1 antigen in two of ten sera from patients with acute bubonic plague and indicated tht antigenaemia in man during plague may reach levels of 4-8 μg of F1 antigen per ml of serum. The probability for a correct serodiagnosis of plague was improved when the patients' sera were tested for both antibody and antigen. Two patients with antigenaemia did not have antibody, while two patients with antibody lacked antigenaemia.

B. Monoclonal Antibodies to Flaviviruses.

1. Heterogeneity of Infection Enhancement of Dengue 2 Strains by Monoclonal Antibodies.

Seven dengue (DEN) 2 virus strains were studied for antibody-dependent enhancement (ADE) of infection in P388D1 mouse macrophage-like cells by using a panel of five DEN 2 monoclonal antibodies. DEN 2 strains were of diverse temporal, geographic, and disease origins. By hemagglutination inhibition and a plague-reduction neutralization test in LLC-MK2 cells, two of the monoclonal antibodies were type specific and three were flavivirus group reactive. In LLC-MK2 cells, the seven DEN 2 viruses each were neutralized by all five monoclonal antibodies. In P388D1 cells, two DEN 2 strains were enhanced by only three monoclonal antibodies, two by four antibodies, and three by all five antibodies, demonstrating that in some instances enhancement is epitope related and not a concentration-dependent function of virus-antibody interactions. However, ADE did not segragate with determinants exhibiting either the flavirus group or the dengue type specificity. The presence or absence of enhancement determinants on DEN 2 strains did not correlate with the geographic origin of virus or the severity of disease yielding the strain. The heterogeneous distribution of enhancement determinants may provide a valence mechanism contributing to a multiple increase of infection enhancement in macrophages.

2. Epitopic Analyses of Dengue Virus Antigens using Monoclonal Antibodies.

Monoclonal antibodies (mAb) prepared against the four dengue virus serotypes were used in competitive binding assays to identify distinct antigenic domains on the (V3) protein of dengue type 2 (DEN-2) virus. Monoclonal antibodies, chosen on the basis of their serological characteristics, were partially purified by either Sephadex-Staph A column chromatograpy or ammonium sulfate salt fractionation and subsequently radiolabeled with ^{125}I. Dilutions of unlabeled monoclonal ascitic fluids were incubated for 12 hours at room temperature with gradient-purified DEN-2 virus absorbed to wells of flexible microtiter plates. Radiolabeled mAb was added and allowed to compete with the unlabeled mAb for 8 to 12 additional hours at room temperature. The use of mAb allowed the identification of the following unique epitopes: type specific (TSa, TSb, TSc), subcomplex-specific (DSCa, DSCb), complex-specific (DCa, DCb), and flavivirus group reactive (FGR). The FGR epitope was positionally distinct from the dengue type-, subcomplex-, and complex-specific domains. However, some type-specific epitopes were contiguous with subcomplex- or complex-specific domains. In some cases the binding of one type of antibody was promoted by a second. The binding of FGR antibodies was promoted by TSc, DSCa, and DCb antibodies. Similarly, TSc antibody binding was promoted by FGR and DSCa antibodies. The interaction of two mutually promoting antibodies (TSc and FGR) was shown to induce protection similar to that of polyclonal DEN-2 hyperimmune fluid in mouse passive protection studies.

3. Viral Specificity of Antibody-Mediated Enhancement of Flavivirus Replication Determined by Monoclonal Antibodies.

Antibody-mediated flavivirus infection of Fc receptor-bearing cells attracted interest because of its possible contribuiton to severe disease associated with sequential dengue infections. The specificity of the antibodies causing the infection-enhancement phemonenon was studied with monoclonal antibodies, and several different flaviviruses and methodologies. Infection enhancement is based on finding increased yields of virus from cells cultured in the presence of subneutralizing concentrations of antibodies as first described by Halstead for enhanced dengue virus infection of human monocytes, and for enhanced yellow fever virus infection of U-937 human monocyte or P388D1 mouse macrophage cell lines (Schlesinger and Brandriss). Infection enhancement can be measured directly if the virus will form plagues on monolayers of macrophages as described for West Nile virus on P388D1 cells (Peiris and Porterfield). In general, it appeared that any monoclonal antibody that binds to the virus also enhances infection. When we used the U-937 human monocyte cell line, dengue-2 virus, and the virus-yield assay system, we found that serotype-specific and flavivirus group-reactive monoclonal antibodies could be clearly distinguished if the monoclonal antibody-virus complexes in the innoculum were formed at a multiplicity of infection below 0.01. Under these conditions, the serotype-specific monoclonal antibodies directed against neutralization and hemagglutination determinants did not enhance virus infection. In contrast, a subcomplex-specific monoclonal antibody reactive only with DEN-1 and DEN-3 enhanced those viruses, and a flavivirus group-reactive monoclonal antibody enhanced DEN-2, 3, 4, but not DEN-1. A dengue complex-reactive monoclonal antibody directed against a polypeptide found only on immature intracellular virions and tested for enhancement only against DEN-2 thus far, was a potent enhancing antibody. IgG, not IgM, enhanced flavivirus infection of Fc receptor-bearing cells, although IgM enhanced infection by some flaviviruses of other cell types under certain conditions. The present data confirm a number of earlier observations that antibodies against cross-reactive, rather than serotype-specific flavivirus antigenic determinants, enhance dengue infection. However, the *in vitro* variables described above (e.g., multiplicity of infection) make it difficult to assess whether serotype-specific IgG would similarly not enhance dengue-2 infection *in vivo*. This question pertains to the use of future vaccines containing only the serotype-specific neutralization determinants.

4. Antibody dependent infection enhancement of P-388D1 Mouse Macrophage-like Cells with Dengue Type 2-derived Monoclonal Antibodies and Wild Dengue Type 2 Strains Isolated from Patients with and without Hemorrhagic Fever.

Using five dengue type 2 (DEN-2)-derived monoclonal antibodies in antibody-dependent enhancement (ADE) assays with P-388D1 cells we have undertaken characterization of 19 DEN-2 viruses isolated from Southeast Asian patients with either dengue hemorrhagic fever (DHF) or uncomplicated dengue. The antibodies were those selected previously by Halstead et al. to characterize seven DEN-2 strains of varied geographic origin by identifying determiants of ADE, neutralization, and hemagglutination inhibition. Three antibodies detected flavivirus group-specific and two detected DEN-2 type-specific determinants. Among hypotheses to be tested are: (a) that wild DEN-2 isolates may be enhanced in a standard *in vitro* system, (b) that one or more enhancement determinants may be heterogeneously distributed among the various isolates, and that identification of such determinants will separate virus strains by association with disease severity, and (c) that by comparison with seven previously characterized DEN-2 strains, determinant-mapping of the 19 wild strains will provide a basis for categorization. Preliminary findings indicate that 13 of 19 wild strains mediate ADE with one or more of five monoclones tested, and that enhancing determinant-mapping sorts the 19 wild isolates into at least four provisional groups. Five wild isolates have the same Group I ADE determinant pattern (all five determinants) as strains TR-1751/16681. Five isolates have the same Group III ADE determinant pattern (three determinants) as the New Guinea C strain, three isolates have a unique two-determinant pattern (Group IV), and six isolates are without detectable ADE determinants. Examination of ADE determinant patterns of the six isolates associated with DHF vs. the 13 associated with uncomplicated dengue suggests that six of six DHF strains vs. seven of 13 other strains had one or more ADE determinants (p=0.06, F.E.T.). Furthermore, three of the DHF strains had the pattern of either the TR-1751/16681 or the New Guinea C strain, and the other three had the unique two-determinant pattern. Thus three of six DHF-associated strains vs. zero of 13 other strains had the Group IV pattern (p=0.02, F.E.T.).

5. Variation in Major Antigens of Japanese Encephalitis Virus Detected by Monoclonal Antibodies.

Hybridomas were prepared by fusing P3x63Ag8.653 myeloma cells with spleen cells from mice immunized with the Nakayama strain of Japanese encephalitis virus (JEV). Animals were primed with suckling mouse brain suspensions of virus and subsequently immunized with either the same form of the virus, purified virion, or detergent-treated virion. Antibody-secreting cell lines were identified with a solid-phase radioimmunoassay using a JEV-infected Vero cell lysate. Of the 300 positive cell lines identified, 55 were chosen for further study and cloned on soft agarose. Antibodies were amplified in ascitic fluids. Monoclonal antibodies were identified as JEV-specific or broadly reactive across the flavivirus group based on hemagglutination-inhibition, plaque-reduction neutralization, and antigen-binding reactions. Among the cross-reactive group were antibodies reacting with some but not all flaviviruses. All antibodies neutralizing virus infectivity reacted by immune precipitation with the V3 envelope glycoprotein. Competitive-binding assays indicated that more than one neutralization epitope exists on the V3 glycoprotein. Certain neutralization epitopes appear to exist as linear sequences as evidenced by the ability of antibodies to react with denatured virion proteins on Western blots. In addition to binding inhibitiion exhibited by antibodies to identical or related epitopes, competitive-binding assays revealed some epitopes responsible for increased binding or "promotion". Mice could be protected from a lethal challenge of JEV by passive administration of certain antibodies. Interestingly, passive administration of non-protective antibodies actually resulted in increased mortality and decreased survival time of mice challenged with sublethal doses of the virus.

Publications:

1. James E. Williams, Mary K. Gentry, Carol A. Braden, Flora Leister, and Robert H. Yolken: Use of an enzyme-linked immunosorbent assay to measure antigenaemia during acute plague. Bulletin of the World Health Organization, 62(3):463-466, 1984.

2. Scott B. Halstead, Chettemgere N. Venkateshan, Mary K. Gentry, and Linda K. Larsen: Heterogeneity of infection enhancement of Dengue 2 strains by monoclonal antibodies. Journal of Immunology, *132*:1529-1532, 1984.

3. E. A. Henchal, W. E. Brandt, D. S. Burke, and M. K. Gentry: Epitopic analysis of dengue virus antigens using monoclonal antibodies. Abstracts of the Annual Meeting of the American Society of Tropical Medicine and Hygiene, 1983, p. 83.

4. W. E. Brandt, J. M. McCown, M. K. Gentry, and E. A. Henchal: Viral specificity of antibody-mediated enhancement of flavivirus replication determined by monoclonal antibodies. Proceedings of the Eleventh International Congress of Tropical Medicine and Malaria, 1984, p. 18.

5. D. M. Morens, S. B. Halstead, D. S. Burke, M. K. Gentry, L. K. Larsen, and W. E. Brandt: Antibody dependent infection enhancement of P-388D1 mouse macrophage-like cells with dengue type 2-derived monoclonal antibodies and wild dengue type 2 strains isolated from patients with and without hemorrhagic fever. Proceedings of the Eleventh International Congress of Tropical Medicine and Malaria, 1984, p. 103.

6. M. K. Gentry, D. Burke, T. Mason, C. Schmaljohn, K. Kopec, and J. Dalrymple: Variation in major antigens of Japanese encephalitis virus detected by monoclonal antibodies. Abstracts of the Sixth International Congress of Virology, 1984, p. 39.

This work unit is being terminated by expiration of the three-year funding period. The technical objective was to produce mouse hybridoma lines secreting monoclonal antibodies to various antigens, to include viruses, bacteria, toxins, enzymes, and peptides. This objective has been accomplished: under this work unit, successful fusions have been done with dengue viruses (serotypes 1,2,3, and 4), *Torpedo* acetylcholinesterase [(13+17)S, 5.6S, and 11S (DFP-treated) species], human serum butytylcholinesterase, fetal bovine serum acetylcholinesterase, plague F1 antigen, Japanese encephalitis virus (Nakayama strain, native, purified virion, and detergent-treated virion forms), Shiga and Clostridium difficile (A and B) toxins, and BCG.

The dengue monoclonal antibodies are distributed throughout the world for use as diagnostic reagents, and we were awarded second prize at the 1982 Army Science Conference for developing these reagents. The Japanese encephalitis antibodies have not been as fully characterized, but at least one antibody has been used in Thailand for diagnostic work, and its distribution is planned. Several JE antibodies are being utilized in cloning the genes coding for the JE V3 glycoprotein. Both the dengue and JE antibodies have been used in establishing topographic maps of their viruses. The plague antibody has proven useful in the detection of antigenemia in clinical disease. Antibodies to the acetylcholinesterase enzyme, the bacterial toxins, and BCG show promise as research tools for antigen mapping.

The following papers and abstracts have been published under this work unit:

1. Gentry, M. K., Henchal, E. A., McCown, J. M., Brandt, W. E., and Dalrymple, J. M.: Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. Am. J. Trop. Med. & Hyg., 31:548-555, 1982.

2. Brandt, W. E., McCown, J. M., Gentry, M. K., and Russell, P. K.: Infection enhancement of dengue type 2 virus in the U-927 human monocyte cell line by antibodies to flavivirus cross-reactive determinants. Inf. & Immun., 36:1036-1041, 1982.

3. Henchal, E. A., Gentry, M. K., McCown, J. M., and Brandt, W. E.: Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am. J. Trop. Med. & Hyg., 31:830-836, 1982.

4. Henchal, E. A., McCown, J. M., Sequin, M. C., Gentry, M. K., and Brandt, W. E.: Rapid identification of dengue virus isolates using monoclonal antibodies in an indirect immunofluorescence assay. Am. J. Trop. Med. & Hyg., 32:164-169, 1983.

5. Griffin, D. E., Gentry, M. K., and Brown, J. E.: Isolation and characterization of monoclonal antibodies to Shiga toxin. Inf. & Immun., 41:430-433, 1983.

6. Doctor, B. P., Camp, S., Gentry, M. K., Taylor, S. S., and Taylor, P.: Antigenic and structural differences in the catalytic subunits of the molecular forms of acetylcholinesterase. Proc. Nat. Acad. Sci., 80:5767-5771, 1983.

7. Henchal, E. A., Brandt, W. E., McCown, J. M., Gentry, M. K., Repic, P. B., and Padmanabhan, R. K.: Recent advances in the molecular biology of the dengue viruses. Proc. Int'l. Conf. on Dengue/Dengue Hemorrhagic Fever, Kuala Lumpur, Malaysia, 1983, pp. 47-57.

8. Halstead, S. B., Venkateshan, C. N., Gentry, M. K., and Larsen, L. K.: Heterogeneity of infection enhancement of dengue 2 strains by monoclonal antibodies. Proc. Int'l Conf. on Dengue/Dengue Hemorrhagic Fever, Kuala Lumpur, Malaysia, 1983, pp. 389-399.

9. Halstead, S. B., Ventakeshan, C. N., Gentry, M. K., and Larsen, L. K.: Heterogeneity of infection enhancement of dengue 2 strains by monoclonal antibodies. J. Immunol., 132:1529-1532, 1984.

10. Williams, J. E., Gentry, M. K., Braden, C. A., and Leister, F., and Yolken, R. H.: Use of an enzyme-linked immunosorbent assay to measure antigenaemia during acute plague. Bull. of the WHO, 62:463-466, 1984.

11. Brandt, W. E., McCown, J. M., Gentry, M. K., and Russell, P. K.: Immune enhancement of dengue-2 virus replication in the U-937 human monocyte cell line by cross-reactive monoclonal antibodies. Fed. Proc., 40:1065, 1981.

12. Henchal, E. A., McCown, J. M., Gentry, M. K., Dalrymple, J. M., and Brandt, W. E.: Evaluation of the serological characteristics of monoclonal antibodies produced against dengue virus antigens. Fed. Proc., 40:1065, 1981.

13. Gentry, M. K., Henchal, E. A., McCown, J. M., and Brandt, W. E.: Development of monoclonal antibodies for identification of dengue virus serotypes. Amer. Soc. Trop, Med. & Hyg., Annual Meeting, 1981.

14. Griffin, D. E., Gentry, M. K., Limrotlert, C. T., Alvarado, M. I., and Fishbein, J. D.: Production and characterization of monoclonal antibodies to Shiga toxin. Amer. Soc. Microbiol., Abs. of the Annual Meeting, 1982., p. 67.

15. Henchal, E. A., McCown, J. M., Gentry, M. K., and Brandt, W. E.: Rapid identification of dengue virus serotypes using monoclonal antibodies in an indirect immunofluorescence test. Army Science Conference, 1982.

16. Henchal, E. A., McCown, J. M., Gentry, M. K., and Brandt, W. E.: Development of monoclonal antibodies for the identification of dengue virus serotypes. Dengue Newsletter, 8:47, 1982.

17. Henchal, E. A., McCown, J. M., Brandt, W. E., and Gentry, M. K.: Antigenic and biological variation occurring among dengue type 4 viruses. Am. Soc. Microbiol., Abstracts of the Annual Meeting, 1983.

18. Doctor, B. P., Camp, S., Gentry, M. K., Taylor, S. S., and Taylor, P.: Evidence for structural and antigenic differences between hydrophobic dimer and asymmetric forms of acetylcholinesterase from Torpedo Californica. Am. Soc. Biol. Chem., 1983.

19. Henchal, E. A.,. Brandt, W. E., Burke, D. S., and Gentry, M. K.: Epitopic analysis of dengue virus antigens using monoclonal antibodies. Abstracts of Am. Soc. Trop. Med. & Hyg., 1983, p. 83.

20. Gentry, M., Burke, D., Mason, T., Schmaljohn, C., Kopec, K., and Dalrymple, J.: Variation in major antigens of Japanese encephalitis virus detected by monoclonal antibodies. Abstracts of the Sixth Int'l. Cong. Virol., 1984, p. 39.

21. Brandt, W. E., McCown, J. M., Gentry, M. K., and Henchal, E. A.: Viral specificity of antibody-mediated enhancement of flavivirus replication determined by monoclonal antibodies. Proceedings of the Eleventh Int'l. Cong. Trop. Med. & Malaria, 1984, p. 18.

22. Morens, D. M., Halstead, S. B., Burke, D. S., Gentry, M. K., and Brandt, W. E.: Antibody dependent infection enhancement of P388D1 mouse macrophage-like cells with dengue type 2-derived monoclonal antibodies and wild dengue type 2 strains isolated from patients with and without hemorrhagic fever. Proceedings of the Eleventh Int'l. Cong. Trop. Med. & Malaria, 1984, p. 103.

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3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY H. Term	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	105 WWJH
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) Neuropharmacology of Performance and Fatigue

12. SUBJECT AREAS
0616 Physiology 0619 Stress Physiology 0510 Psychology 0615 Pharmacology

13. START DATE 81 10
14. ESTIMATED COMPLETION DATE 84 10
15. FUNDING ORGANIZATION DA
16. PERFORMANCE METHOD C. In-House

17. CONTRACT GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	[illegible]
85	0.0	0

19. RESPONSIBLE DOD ORGANIZATION
a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100
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20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
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c. NAME OF PRINCIPAL INVESTIGATOR Belenky, GL
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f. NAME OF ASSOCIATE INVESTIGATOR (if available) Holaday, JW
g. NAME OF ASSOCIATE INVESTIGATOR (if available) Tortella, FC

21. GENERAL USE FINA
MILITARY/CIVILIAN APPLICATION H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Performance (U) Pharmacology (U) Stress (U) Stimulant (U) Fatigue (U) Sleep (U) Perseveration (U) Lab Animals (U) rats

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23.(U) Initial animal studies evaluating the role of endogenous and exogenous neuromodulators in performance, fatigue and sleep have been completed. Stimulant drugs which are presently available (e.g. amphetamine) have the adverse consequence of severely impariing judgement while providing their stimulant effects. Insights into the body's own systems for producing arousal, sleep and other behaviors relating to vigilance and performance have suggested the use of novel substances that may act either directly or indirectly to enhance endogenous stimulants and depress endogenous sedatives. This knowledge will suggest new approaches to maximizing performance on the battlefield.

24.(U) Experimental animals were evaluated for alterations in locomotor activity and performance before and after treatment with various drugs which affect performance (including thyrotropin releasing hormone (TRH), amphetamine, prochlorperizine and opioid agonists and antagonists) or treatment with electroconvulsive shock (ECS). Correlations among behavioral findings, analysis of biochemical substances and assay of receptor changes allowed for interactive evaluations. Recently acquired electroencephalographic instrumentation provided an opportunity to correlate changes in brain activity with the behavioral responses.

25.(U) 83-10 - 84 09 Electroconvulsive shock resulted in a significant increase in the number of binding sites for various drugs, including opioid compounds. The increased number of opioid receptors was shown to occur only in the absence of Sodium and to be discretely localized in areas of the brain involved in cognition and behavior. Studies with Dr Kornetsky (Boston U. School of Medicine) indicated that TRH enhanced reward responses in rats. From the interest evoked by these experiments, a new study group was initiated among members of the Division of Neuropsychiatry to investigate promising candidates for enhancing human performance. A conference was organized by this group entitled "Pharmacological Optimization of Performance" featuring international authorities in the areas of psychopharmacology and psychophysiology. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project: 3A161101A9C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 105

Neuropharmacology of
Performance and Fatigue

Investigators:
Principal: Belenky, G.L., M.D.
Associate: Holaday J.W., Ph.D., Tortella, F.C., Ph.D.

Objectives:

Stimulant drugs which are presently available (e.g. amphetmine) have the adverse consequence of severely impairing judgement while providing their stimulant effects. Insights into the body's own systems for producing arousal, sleep and other behaviors relating to vigilance and performance have suggested the use of novel substances that may act either directly or indirectly to enhance endogenous stimulants and depress endogenous sedatives. Application of this knowledge may help to develop new approaches to maximizing battlefield performance.

Approach:

Experimental animals were evaluated for alterations in locomotor activity and performance before and after treatment with various drugs which affect performance (including thyrotropin releasing hormone (TRH), amphetamine, prochlorperizine and opioiod agonists and antagonists) or treatment with electroconvulsive shock (ECS). Correlations among behavioral findings, analysis of biochemical substances and assay of receptor changes allowed for interactive evaluations of behavioral and biochemical correlates. Recently acquired electroencephalographic instrumentation provided an opportunity to correlate changes in brain activity with the behavioral responses. Knowledge from these experiments provided an opportunity to assess new directions for future research.

Progress:

Electroconvulsive shock resulted in a significant increase in the number of binding sites for various drugs, including opioid compounds. The increased number of opioid receptors was shown to occur only in the absence of Sodium and to be discretely localized in areas of the brain involved in cognition and behavior. Collaborative studies with Dr. Kornetsky (Boston U. School of Medicine) indicated that TRH enhanced reward responses in rats. From the interest evoked by these experiments, a new study group was initiated among members of the Division of Neuropsychiatry to investigate promising pharmacological candiates for altering human performance.

To further this effort, a conference was organized by this group entitled "Pharmacological Optimization of performance" featuring international authorities in the areas of psychopharmacology and psychophysiology.

This research was supported because of the need to establish new ways to enhance performance and prevent fatigue. Presently available stimulant drugs such as amphetamine have the adverse consequence of impairing judgement, thus severely limiting their utilitiy in military and non-military environments. If insights into the body's own systems for producing arousal, sleep and other behaviors relating to vigilance and performance are elucidated, it may be possible to augment responses which elicit arousal or, conversely, to block systems which contribute to fatigue. Even if no such mechanisms are elucidated, the investigations with new drugs and neuropeptide hormones which have recently become available may provide pharmacological tools to enhance performance, delay fatigue and sleep and have minimal adverse side effects.

For additional information see 1984 Annual Report, Project 3E162777A8 79.

AVAILABLE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 303104

2. DATE OF SUMMARY: 84 10 01

REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	106 WWJS
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) Stress Factors Among Senior Non Commissioned Officers

12. SUBJECT AREAS
0605 Clinical Medicine 0510 Psychology

13. START DATE: 83 10

14. ESTIMATED COMPLETION DATE: CONT

15. FUNDING ORGANIZATION: DA

16. PERFORMANCE METHOD: C. In-House

17. CONTRACT GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	1.0	50
85	1.0	25

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL: TOP, F H JR
d. TELEPHONE NUMBER (include area code): (202)- 576-3551

20. PERFORMING ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS
Division of Neuropsychiatry
Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR: INGRAHAM, L H
d. TELEPHONE NUMBER (include area code): (202)- 427-5360
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE
FINA
MILITARY CIVILIAN APPLICATION H

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) STRESS; (U) NCO; (U) ~~HUMAN~~ VOLUNTEERS

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The study examines stress factors among senior non-commissioned officers. The objective is to understand the stresses and strains in the NCO role which contribute to individual and organizational dysfunction. This research is of military importance.

24. (U) The method is narrated career histories which is a modification of life history techniques used in social anthropology, sociology, and oral history.

25. (U) 8310-8409 Note prior title: Acquisition of NCO skills for Stress Casualty Prevention. Sixteen of 24 interviews have been completed; four interviews have been abstracted; one interview has been transcribed. Preliminary analysis suggests the following themes for analysis: (1) The meaning of "NCO" is ambiguous since level of responsibility, level of technical skill, and longevity are frequently confounded. (2) NCO ranks differ qualitatively in the nature and kind of authority inherent in them. (3) Leadership modes change across NCO ranks. (4) "Taking care of soldiers" encompasses all "NCOs," regardless of technical specialty or expertise. (5) Commissioned and non-commissioned officers differ both in the scope and kind of work performed. (6) Confusion exists regarding the duties, respondibilities, and authority of sergeants major. (7) NCOs use different ethical standards than do officers. (8) Commissioned and non-commissioned officer relationships change across their respective ranks. (9) The issue in contention between senior commissioned and non-commissioned officers is whether and how NCOs are "professionals." Interviewing, abstracting, transcribing, and analysis continue. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

Project: 3A161101A91C In-House Laboratory Independent Research

Work Unit: 106 Stress Factors Among Senior Non-commissioned Officers

Principal Investigator: LTC Larry H. Ingraham, MS

Problem and Objectives:

This project stemmed from the observation, most recently confirmed by Israeli forces in Lebanon, that the single most critical element in the prevention and management of combat stress casualties is the quality of the interpersonal relations in face-to-face work groups. "Unit cohesion" efforts like the New Manning System are attempting to stabilize membership in these groups, but it is non-commissioned officers who are the central figures in the creation and maintenance of the groups' interpersonal climate. The goal of this work is to discover how (or if) they come to learn their role in this process.

Progress:

Inquires at the Defense Technical Information Center, the Army Library, the Army Sergeant Major's Academy, and the Marine Corps Oral History Center turned up absolutely no literature on the topic, nor did a survey of the civilian literature in the field of military sociology. The work unit thus proposed to start with NCO's themselves, using the methods of the oral historian: recording career histories of senior non-commissioned officers. Through recall of concrete events and actual people, a data base can be established focusing on what NCO's at various levels actually do, what aspects of those activities can be learned in school, and how they develop and apply standards of conduct, discipline and morale in actual practice.

Sixteen of a projected 24 histories have been collected (on tape); four have been abstracted; one has been transcribed. The clearest finding to date has been that even successful NCO's have rarely if ever seen or been shown their role in preventing stress casualties. In fact the dominant theme emerging has been the great stress imposed on the NCO Corps by the lack of a clear and lasting definition of "sergeants work" at all. Preliminary analysis has suggested the following themes for analysis: (1) The meaning of "NCO" is ambiguous since level of responsibility, level of technical skill, and longevity are frequently confounded. (2) NCO ranks differ qualitatively in the nature and kind of authority inherent in them in them. (3) Leadership modes change across NCO ranks. (4) "Taking care of soldiers" encompasses all "NCOs," regardless of technical speciality or expertise. (5) Commissioned and non-commissioned officers differ both in the scope and kind of work performed. (6) Confusion exists regarding the duties, responsibilities, and authority of sergeants major. (7) NCOs use different ethical standards than do officers. (8) Commissioned and non-commissioned officer relationships change across their respective ranks. (9) The issue in contention between senior commissioned and non-commissioned officers is whether and how NCOs are "professionals."

This project was supported because preventing battle stress casualties, like preventing cold or heat inquiries is a joint responsibility of medical and line personnel. The former advise, but the latter must see the preventive actions through. If these first level supervisors fail to see this role or see it differently than do the medical personnel, it is the latter who will bear the burden of treating unnecessary casualties. This project should be completed in FY85.

Presentation:

Ingraham, L.H. Is the Army ready for smart sergeants? Address to the TRADOC Command Sergeants Major Conference, El Paso, June 21, 1984.

Publication: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION DA 303105	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX
9. LEVEL OF SUM A. WORK UNIT					

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	WWL6 107
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) Neogut

12. SUBJECT AREAS
0616 Physiology 0602 Bioengineering

13. START DATE 83 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
c. CONTRACT/GRANT NUMBER
d. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	3.0	30
85	3.0	30

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Division of Surgery Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR HARMON, J W
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-3791
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) [illegible] g. NAME OF ASSOCIATE INVESTIGATOR (if available) BASS, B; GARCIA, V

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) small bowel; (U) nutrition; (U) short gut syndrome; (U) fetal rat; (U) Lab animals

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23(U) The primary objective is to study means to augment the absorptive area of the small intestine to provide nutrition for individuals with "short gut syndrome." Currently if the small bowel is lost secondary to trauma, internal hernia or diseases such as Crohn's disease, enteral nutrition is impossible. Such patients must be maintained forever on intravenous infusion pumps which limits activity, is expensive and difficult and sometimes technically impossible. There is military relevance in this research.

24(U) The approach will be to transplant intestine from fetal Fisher rats into the subcutaneous space of adult Fisher rats. Such intestine becomes vascularized, enlarges, and may develop functions of normal intestine.

83 10-84 09

25(U) Successful transplants of fetal rat small bowel to isogeneic adult Fisher rats have been carried out and the resulting "neogut" has been shown to have typical gross and microscopic appearance of small bowel. In addition this neogut has been shown to preferentially transport glucose from mucosa to serosa in Ussing Chambers and to have electrophysiologic properties of transmucosal electrical potential difference and short circuit current similar to normal rat small bowel. This material was presented to the American College of Surgeons, Surgical Research Forum and a manuscript has been accepted by the Annals of Surgery. Further projects are underway to evaluate angiogenesis and to attempt to transplant larger size bowel as would be required in man. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project: 3A162202A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit: 107 Neogut

Investigators:
Principal: John W. Harmon, LTC, MC
Co-Investigators: Barbara Bass, CPT, MC
Jonathan Jaffin, CPT, MC
Dennis Mortiz, MAJ, MC
Rogery Perry, CPT, MC

Background and Objectives:

"Short gut" syndrome is a condition where patients have lost sufficient small intestine to be unable to maintain their nutrition through external feeding. It can result from any condition which causes small intestinal loss including trauma, obstruction, mesenteric thrombosis, and necrotizing enterocolitis. These patients are currently kept alive using parenteral nutrition, which is expensive and subjects these patients to a host of life threatening metabolic and septic complications.

To avoid these complications, investigators have attempted to increase the bowel surface area with pedicle flaps, dacron patches, and colonic serosa grafts. These have resulted in only small increases in the absorptive area of the small intestine.

Recently, an alternative method of expanding small bowel absorptive area has been described. This involves transplanting avascular fetal intestinal tissue into the subcutaneous tissue of host animals. This has been described as growing into bowel-like structures. This method is being studied at WRAIR. Initial objectives involve performing detailed anatomic and physiologic studies to characterize the transplanted tissue, called "neogut". Further objectives include developing ways to improve the growth of neogut and to eventually feed animals through the neogut.

Progress. Anatomic Studies: During this fiscal year, techniques were devised for reliable transplantation of neogut. These resulted in a greater than 70% successful transplantation rate in Fisher rats. The neogut had the external appearance of distended bowel. There was a dense fibrous reaction around the bowel, with striking neovascularization. The internal wall of the neogut had the appearance of normal musosa. Histologic examination demonstrated the neogut to be similar to rat small intestine as did scanning and transmission electron microscopy. The neogut was shown to transplant glucose.

These results demonstrate that avasuclar segments of fetal rat intestine transplanted to the subcutaneous tissues of syngeneic host animals will become vascularized, grow and develop certain properties of normal intestine. This "neogut" has structural similarities to normal small bowel including an intact epithelium of mixed cellularity, lamina propria, and bilauer muscularis. Villus structure in many specimens is altered relative to normal intestine; flattened and cobblestone-like mucosa may be present. Additionally, microvilli are less dense and generally not as long as in normal intestine. These alterations may be related to intralumimal pressure that develops in these chronically obstructed intestinal segments which actively secrete mucus and shed dead epithelium into the lumen. These secretions cause ever increasing distension of the bowel. Additionally, these loops have never been exposed to nutrients. This disuse may contribute to the shortened, sparse microvilli observed with electron microscopy.

The present study is the first to assess the motility of transplanted fetal intestine. The myoelectric parameters observed suggest that the smooth muscle in neogut has functional characteristics similar to normal ileum but lacks mature neuronal control. Instrinsic myogenic mechanisms are responsible for slow wave generation. The slow wave frequency of neogut is slightly less than that of ileum; this may reflect a lack of extrinsic innervation. A similar reduction in slow wave frequency has been reported in extrinsically denervated rabbit ileal loops studies in vitro. The presence of spike activity, particularly action potential complexes, suggests that neogut smooth muscle is also capable of contraction.

This method of transplantation has several merits that may make it superior to conventional vascularized small intestinal allografts. First, the technical difficulties of vascular anastomoses are eliminated. Second, the lymphoid tissue in fetal intestine may be more tolerant of foreign antigens minimizing graft versus host reactions. Third, the mass of lymphoid tissue transplanted is much smaller than in conventional allografts, also minimizing the risks for graft versus host disease. Fourth, the widespread practice of voluntary abortion creates the potential for ready availability of transplantable tissue. Fifth, fetal intestinal tissue is not colonized with enteric organisms, minimizing potential septic complications.

There are several difficulties and unanswered questions regarding the applicability of neogut to human clinical use. Rejection phenomena were avoided in this study by using syngeneic rat strains. Schwartz, using this transplantation technique in rats, found a 100% rejection rate of transplants performed across genetic barriers. In that study, Cyclosporin A was effective in improving graft survival to 70% in cross strain transplants. No graft versus host disease was reported. Multiple strictures, most likely related to irregular neovascularization, may also limit the usefulness of this tissue. Technical changes to improve vascularization such as implanting the intestine in abdominal wall musculature or in omental tubes, may minimize this limiting complication. The more subtle myoelectric and electrophysiologic differences between neogut and normal intestine may be readily revesible, or preventable, with early decompression of the segments allowing luminal perfusion with nutrients.

The structural and physiologic similarities of neogut to normal rat small bowel suggest this tissue may be able to provide a nutritionally useful accessory gut. Refinement of techniques to maximize neovascularization, to control rejection phenomena and to allow luminal perfusion and decompression of the segments following transplantation may make this technique of value to the patient with short-gut syndrome.

Recommendations for the future:

1. We are currently working on ways to increase vascularity of the neogut and also to place it in positions more accessible to the intestinal tract.
2. We are also working on methods to feed animals through their neogut with continuous infusion pumps.
3. We are also working on transplanting rabbit neogut to athymic rats.

Publications:

Bass et al. Annals of Surgery accepted for publication.

DA 303100

DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	108 WWHC
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE *(Precede with Security Classification Code)*
(U) Inhibitors of Methylation as Potential Therapeutic Agents

12. SUBJECT AREAS
0601 Biochemistry 0603 Biology 0615 Pharmacology

13. START DATE 83 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-house

17. CONTRACT GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	0.5	20
85	0.5	25

19. RESPONSIBLE DOD ORGANIZATION

a. NAME Walter Reed Army Institute of Research

c. ADDRESS *(include zip code)*
Washington, DC 20307-5100

c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H Jr

d. TELEPHONE NUMBER *(include area code)*
(202)-576-3551

21. GENERAL USE FINA

MILITARY/CIVILIAN APPLICATION H

20. PERFORMING ORGANIZATION

a. NAME Walter Reed Army Institute of Research
Division of Biochemistry

b. ADDRESS
Washington, DC 20307-5100

c. NAME OF PRINCIPAL INVESTIGATOR
Chiang, P K

d. TELEPHONE NUMBER *(include area code)*
(202)-576-1361

f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
Gordon, R K

g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
Doctor, B P

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Therapeutic Agents; (U) Methylation; (U) S-adenosylhomocysteine; (U) S-adenosylmethionine

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The objective of this work unit is to study the role of inhibitors of methylation reactions as potential therapeutic agents: in viral, parasitic infection and as inducers of cellular differentiation. There is military relevance in this research.

24. (U) The potential chemicals to be screened for their ability to inhibit methylation are nucleosides; specifically, nucleosides that are potent inhibitors of S-adenosylhomocysteine hydrolase. Nucleosides that are potent inhibitors and/or alternative substrates of S-adenosylhomocysteine hydrolase will be screened for their ability to raise the cellular level of S-adenosylhomocysteine and/or S-nucleosidylhomocysteine. The formation of nucleocidinylhomocysteine normally leads to the inhibition of methylation reactions. The systems to be examined for the biological consequences are: HL-60 promyelocytic leukemia cells, viruses, Plasmodium in culture, and the differentiation of 3T3-L1 fibroblasts to fat cells.

25. (U) 83 10 - 84 09: HL-60 human promyelocytic leukemia cells have been found to undergo differentiation to monocytes and granulocytes in the presence of 3-deaza-(±)aristeromycin, neplanocin A (a potent antitumor agent), and phorbol ester. The differentiation is accompanied by changes in metabolites of methylation. Plasmodium cultures are potently inhibited in growth by neplanocin A, and also 3-deaza-(±)-aristeromycin, regardless whether they are resistant strains or not. Some of the new adenosine analogs have also been found to exhibit anti-viral activity in vitro. In vivo tests are being conducted.

PROJECT: 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

WORK UNIT: 108 Inhibitors of Methylation as Potential Therapeutic Agents

INVESTIGATORS:

Principal: Peter K. Chiang, Ph.D.
Associate: Richard K. Gordon, Ph.D.; SP6 George Miura Ph.D.;
Jarle Aarbakke, M.D., Ph.D.; John A. Duerre, Ph.D.
Assistant: SP4 Felipe N. Padilla, Michelle M. Richard

The objective is to synthesize and identify specific inhibitors of methylation as potential agents: in viral, parasitic infection and as inducers of cellular differentiation.

The potential chemicals to be screened for their ability to inhibit methylation are nucleosides; specifically, nucleosides that are potent inhibitors of S-adenosylhomocysteine hydrolase. Nucleosides that are potent inhibitors and/or alternative substrates of S-adenosylhomocysteine hydrolase will be screened for their ability to raise the cellular level of S-adenosylhomocysteine and/or S-nucleosidylhomocysteine. In the event of the latter, S-adenosylmethionine will also be increased as a consequence of the inhibition of methylation reactions caused by the appearance of large amounts of S-adenosylhomocysteine and S-nucleosidylhomocysteine. The changes in the cellular levels of these metabolites will be analyzed by high pressure liquid chromatography. The specific biological targets to be looked at will be viruses, Plasmodium cultures and cell lines that can be induced to undergo differentiation. The cell lines to be examined are 3T3-L1 fibroblasts that can under differentiation to become fat cells, and HL-60, which are human promyelocytic leukemia cells that can differentiate into more mature granulocyte-like cells. Correlative changes in DNA methylation, RNA methylation, lipid methylation, and protein methylation will be studied.

1. Induction of differentiation of HL-60 promyelocytic leukemia cells:

3-Deazaaristeromycin (dzAri) and 3-deazaguanosine (dzGuo), potent inhibitors of adenosylhomocysteine (AdoHcy) hydrolase (AdoHcyase) and IMP-dehydrogenase, respectively, induce differentiation in the human promyelocytic cell line HL-60 to acquire morphological and functional characteristics of neutrophils. Since gene expression and cellular differentiation have been associated with undermethylation of DNA, and since cellular methylation is influenced by endogenous AdoHcy and AdoMet, their concentrations were measured in cells treated with 3-deaza nucleosides. After 8 h, cells treated with 5)M dzAri showed a 138% increase in AdoMet, whereas cells exposed to 3)M dzGuo had 178% increase in AdoHcy. There was a dose-dependent increase in AdoHcy caused by dzAri, with concomitant decreases in cell proliferation. In contrast, 3)M dzGuo was without any effect on cell number and viability after 48 h. Because it takes 3-7 days to induce differentiation in HL-60 cells by the 3-deaza nucleosides, the increase in the cellular AdoHcy/AdoMet ratio in the presence of both 3-deaza nucleosides at 8 h is an early event associated with differentiation. Even though dzGuo has no known effects *in vitro* on isolated AdoHcyase, it may induce HL-60 differentiation by affecting DNA undermethylation via a mechanism similar to dzAri.

2. Differentiation of HL-60 Human Promyelocytic Cellls Induced By 3-Deazanucleosides and Phorbol Ester.

3-Deazaaristeromycin (dzAri) and 3-deazaguanosine (dzGuo), potent inhibitors of adenosylhomocysteine (AdoHcy) hydrolase and IMP-dehydrogenase, respectively, induce differentiation of HL-60 cells. Since undermethylation of DNA leads to gene expression, and may be controlled by AdoHcy/AdoMet ratio, the latter metabolites were measured. AdoHcy/AdoMet increased significantly in the cells treated by dzAri and dzGuo, as early as 8 h. Additionally, initial studies showed that in cells induced by phorbol-12-myristate-13-acetate (PMA), the binding of human IgG complexes to Fc receptors increased to 11 x 10^3 from 3 x 10^3 molecules/cell in controls. Moreover, the uptake of the protozoan *Leishmania tropica* in its amastigote form was observed in 20% of the induced cells, in sharp contrast to less than 1% in the uninduced cells. Whether PMA induces differentiation via mechanisms similar to the 3-deazanucleosides remains to be elucidated.

3. Perturbation of S-adenosylmethionine, polyamine and catecholamine metabolism by 3-deazaadenosine:

Using a new high performance liquid chromatography technique which permits the analysis of a variety of sulfur metabolites including methylthioadenosine, the effects of 3-deazaadenosine (dzAdo), a potent inhibitor of AdoHcy hydrolase, were examined in male rats and HeLa cells. Intraperitoneal injection of dzAdo to rats increased the liver concentrations of AdoMet and AdoHcy by 1.7- and 2.7-fold, respectively. In contrast, levels of AdoMet and AdoHcy in the adrenals were not altered. However, there was a formation of dzAdoHcy. Also, in the adrenals there was a 50% inhibition of the methylation of norepinephrine to epinephrine, accompanied by a 60% decrease in L-dopa but a 220% increase in dopamine. When HeLa cells were equilibrated in [^{35}S] methionine and then incubated 90 min with dzAdo, increases in cellular AdoHcy and AdoMet were also observed. Concomitantly, there was a drop in cellular decarboxylated AdoMet, thus corroborating the inhibitory effect of dzAdo in AdoMet decarboxylase. In addition putrescine accumulated, along with drops in cellular spermidine and spermine. These data demonstrate some of the metabolic consequences that result from the inhibition of AdoHcy hydrolase and AdoMet decarboxylase by dzAdo.

4. Analysis of S-Adenosylmethionine and Related Sulfur Metabolites in Animal Tissues.

A high-performance liquid chromatographic method was devised that separates S-adenosylmethionine and related sulfur metabolites on a Radial-PAK SCX cation-exchange column using a four-step $NH_4COOH/(NH_4)_2SO_4$ elution gradient. This new procedure permits, in a single run of 60 min, the quantitative analysis of S-adenosylmethionine, S-adenosylhomocysteine

(AdoHcy), 5'-deoxy-5'-methylthioadenosine, decarboxylated S-adenosylmethionine, decarboxylated S-adenosylhomocysteine, inosylhomocysteine, and other related metabolites. Furthermore, this method allows the detection in rat tissues of novel sulfur metabolites, S-inosylhomocysteine and decarboxylated S-adenosylhomocysteine. Perturbation of the levels of some of these metabolites could be detected in rat livers and spleens after the administration of 3-deazaadenosine, an inhibitor of adenosylhomocysteine hydrolase, but could not be detected in rat adrenal glands. It is notable that decarboxylated AdoHcy disappeared in the livers of rats treated with 3-deazaadenosine. HeLa cells incubated with [^{35}S]methionine displayed the incorporation of the labeled sulfur into S-adenosylmethionine, S-adenosylhomocysteine, decarboxylated S-adenosylmethionine, S-inosylhomocysteine, and 5'-deoxy-5'-methylthioadenosine.

5. Antimalarial Activity of SIBA and deaza-SIBA:

Biological methylation reactions have been thought to be essential for cell growth and differentiation. In a study to elucidate the relation between methylation and polyamine biosynthesis, employing the in vitro cultures of P. falciparum-infected human red cells, we have examined two adenosine analogues thought to be methylation inhibitors, viz. 5'-deoxy-5'-isobutylthio-3-adenosine (SIBA) and 5'-deoxy-5'-isobutylthio-3-deazaadenosine (deaza-SIBA) for growth and polyamine biosynthesis. Growth was assessed by monitoring [^{3}H]hypoxanthine incorporation into RNA, DNA and protein, as well as by evaluating parasite morphology and numbers of Giemsa-stained blood films. Protein-free supernatant fluids from red cell pellets and extracellular fluid were analyzed for polyamines by an ultramicro automated HPLC method. Both SIBA and deaza-SIBA inhibited intraerythrocytic parasite growth: the former inhibited 96% at 0.3mM (confirming Trager, 1978), and the latter inhibited 74% at 0.1 mM. Morphologically, there was an arrest at the ring stage with decreased numbers and distorted morphology, as well as pyknosis of parasite nuclei of other intraerythrocytic parasite stages. In addition, red cells in parasitized cultures showed echinocyte formation. Polyamine levels in both SIBA and deaza-SIBA treated red cells showed increases in putrescine and spermidine in both parasitized and unparasitized cultures. Hypoxanthine (1 uM) added 24 h prior to harvest, appeared to improve parasite morphology and reverse elevations of spermidine and spermine induced by the drugs. However, parasite counts were not significantly increased.

6. Biological Activity and Synthesis of an Isomer of Formycin: Biological Activity and a Modified Synthesis of 8-Amino-3-(b-D-Ribofuranosyl)-1,2,4-Triazolo[4,3-a]Pyrazine, An Isomer of Formycin.

A two step synthesis of of 8-amino-3-(b-D-ribofuranosyl)-1,2,4-triazolo[4,3-a]pyrazine (1) has been devised. This compound is (i) a very poor substrate for adenosine deaminase, (ii) both a competitive and irreversible inhibitor of S-adenosylhomocysteinase in the synthesis direction, (iii) can inhibit L1210 cell growth in culture, (iv) inhibits both the cellular uptake of nucleic acid precursors and their incorporation into the nucleic acids of L1210 cells, (v) is inert to metabolism to its 5'nucleotides, and (vi) is a weak antiviral agent and coronary vasodilator.

7. Neplanocin A: Effects of Putrescine and S-Adenosylmethionine Metabolism in Malaria-Infected Human Red Cell Cultures.

Neplanocin A, a novel carbocyclic analogue of adenosine, with a cyclopentene moiety replacing the ribose, has been known to be an effective antitumor agent. As part of a study to evaluate drugs that will inhibit malaria parasites, Neplanocin A was found to be effective as an antimalarial with ED_{50} of 3.1)M and 2.6)M, respectively, for FCR-3/Gambia and Camp/Malay strains of *Plasmodium falciparum*. Because Neplanocin is a potent inhibitor of S-adenosylhomocysteine hydrolase, and possibly, methylation reactions, the mechanism of the anti-malarial effects was explored by examining the levels of metabolic intermediates that were altered. In infected cells, S-adenosylmethionine levels rose and reached a plateau at levels greater than 1)M Neplanocin A, while S-adenosylhomocysteine levels peaked at 2.5)M drug. Two putative Neplanocin A metabolites increased in a dose-dependent manner. In control uninfected red blood cells, the perturbation of all intermediates was the same. Cellular contents of putrescine in cultures treated 48 h with 5)M drug rose, whereas spermidine and spermine levels remained unchanged. Morphologically, at drug doses over 2.5 to 5)M, both infected and uninfected red cells appeared to undergo echinocyte formation. These results suggest that Neplanocin A is an antimalarial that acts by the inhibition of S-adenosylhomocysteine hydrolase which in turn inhibits a variety of methylation reactions necessary for normal parasite functions, and in addition, affects putrescine metabolism.

8. Biological Effects of Neplanocin A with Associated Changes in Cellular Levels of S-Adenosylhomocysteine and S-Adenosylmethionine.

Neplanocin A (NPC), a novel cyclopentene analog of adenosine with antitumor activity, is a potent inhibitor of S-adenosylhomocysteine (AdoHcy) hydrolase with a K_i of 3 nM. When tested in cells, NPC caused drastic increases in AdoHcy in a dose-dependent manner, concomitant with the formation of a putative new metabolite, S-neplanocinyl-methionine. In most cases, there was a drop in S-adenosylmethionine. There are interesting biological effects that were caused by NPC. After treatment with NPC, HL-60 human promyelocytic leukemia cells were induced to differentiate in micromolar concentrations, acquiring morphologic features of neutrophils. NPC is also an effective anti-malarial when tested against FCR-3/Gambia and Camp/Malay strains of *Plasmodium faciparum* with ED_{50} pf 3)M. Morphologically, both infected and uninfected red cells appeared to undergo echinocyte formation between 2.5 to 5)M. In addition, cellular level of putrescine in red cells increased without changes in spermidine and spermine.

PUBLICATIONS

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Miura, G. A., Santangelo, J. R., Gordon, R. K., and Chiang, P. K.: Analysis of S-adenosylmethionine and related sulfur metabolites in animal tissues. Anal. Biochem., 141, 161-167, 1984.

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Chiang, P. K., Lucas, D. L., and Wright, D. G.: Induction of differentiation of HL-60 human promyelocytic leukemic cells by 3-deazapurines. Ann. N. Y. Acad Sci., in press.

ABSTRACTS

Aarbakke, J., Finbloom, D. S., Miura, G. A., Nacy, C. A., and Chiang, P. K.: Differentiation of HL-60 human promyelocytic cells induced by 3-deaza-nucleosides and phorbol ester. IUPHAR 9th International Congress of Pharmacology (1984).

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Whaun, J. M., Miura, G. A., Brown, N. D., and Chiang, P. K.: Neplanocin A: Effects on putrescine and S-adenosylmethionine metabolism in malaria-infected human red cell cultures. International Conference on Polyamines; Budapest, Hungary (1984).

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Miura, G. A., Gordon, R. K., Brown, N. D., Rush, R. S., Padilla, F. N., and Chiang, P. K.: Perturbation of S-adenosymethionine, polyamine and catecholamine metabolism by 3-deazaadenosine. Fed. Proc. 43, 3542 (1984).

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Whaun, J. M., Miura, G. A., Brown, N. D., and Chiang, P. K.: Neplanocin A: Effects on putrescine and S-adenosylmethionine metabolism in malaria-infeted human red cell cultures. International Conference on Polyamines; Budapest, Hungary (1984).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 303107	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	109 WWHD
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) Elucidation of Antigenic Determinants

12. SUBJECT AREAS
0613 Microbiology 0703 Organic Chemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM. TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	0.5	80
85	1.5	80

19. RESPONSIBLE DOD ORGANIZATION
a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
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d. TELEPHONE NUMBER (include area code)
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
Division of Biochemistry
b. ADDRESS
Washington, DC 20307-5100
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Gentry, M K
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(202)-576-3001
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
Doctor, B P
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
Rush, R S

21. GENERAL USE FINA

MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Monoclonal; (U) Peptide; (U) Antibody: (U) Determinants

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objective of this project is to elucidate antigenic determinants of infletious agents and other macromolecules against which monoclonal antibodies are directed so that synthetic peptide vaccines can be produced and tested for bioefficacy. There is military relevance in this research.

24. (U) Purified monoclonal antibody against given protein or peptide antigens will be coupled to a solid support in such a fashion that Fab region is exposed for binding to antigenic determinants. The antigens will be digested with trypsin or chymotrypin or other proteolytic enzymes. The Digest will be subjected to affinity chromatography on monoclonal antibody column. The effluent peptide map will be compared with original peptide map by HPLC. The differential peptide will be purified, its amino acid sequence determined and a series of peptides varying one amino acid will be synthesized. These peptides will be tested for their antigenicity and protective activity.

83 10 - 84 09

25. (U) Monoclonal antibody against human erythrosite acetylcholinesterase was purified and successfully coupled to protein A sepharose. Using the combination of affinity chromatography and an HPLC map of tryptic digested AchE, the antigenic peptide(s) has been identified. It will be sequenced. This monoclonal antibody cross reacts with bovine and Torpedo acetylcholinesterase thus may identify a common antigenic determinant in all AchEs. The nona peptide which constitutes a cyclic AMP binding site on catalytic subunit of protein Kinase was successfully encapsulated into lyposomes and used for mouse injection. Immunoassay and western blots of ascitic fluid indicate presence of antibody. Topography of this antigenic determinant on protein kinase will be elucidated.

PROJECT: 3A161101191C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

WORK UNIT: 109 Elucidation of Antigenic Determiants

INVESTIGATORS:

Principle:	Mary K. Gentry, B.S.
Associate:	B. P. Doctor, Ph.D.; Robert S. Rush, Ph.D.; SP4 Karla K. Kopec, B.S.; SP4 Marcia K. Hamman
In collaboration with:	Palmer Taylor, Ph.D.; L. B. Howland, Ph.D.; Mark Ellisman, Ph.D.

DESCRIPTION:

Antigenic determinants of infectious agents and macromolecules of military relevance against which monoclonal antibodies have been produced are being studied. Techniques include purification of monoclonal antibodies and their use in affinity chromatography. Antigens collected on affinity columns are subjected to digestion and peptide mapping. Synthetic peptides based on the original sequences can be used for studies on anti-genicity and protection and, ultimately, in the development of synthetic peptide vaccines.

A. Molecular Studies on Acetylcholinesterases.

1. Molecular and Structural Basis for the Polymorphic Forms of Acetylcholinesterase.

Acetylcholinesterase in various tissues is found to exist as two general classes of molecular forms. The first consists of dimensionally asymmetric forms composed of multiple catalytic subunits disulfide-linked to collagen-like tail unit and, in some cases, a noncollagenous structural subunit. These forms are found in highest abundance in synaptic areas and their biosynthesis is controlled by synaptogenesis. The second class consists of simple oligomers of the catalytic subunits. These forms are often hydrophobic, associate with membranes, and show a wide tissue distribution extending to both excitable and non-excitable tissue. In muscle, these forms are present in both junctional and extrajunctional areas.

Three explanations which are not mutually exclusive might be put forward as the bases for variegation in acetylcholinesterase molecular structure. The acetylcholinesterases may be expressed as a multigene family where the primary sequences of the catalytic subunits of the molecular forms differ. Second, the structural differences could arise from a single gene and alternative m-RNA processing would cause the structural diversity. Third, differences in structure could arise from posttranslational modifications giving rise to different extents of glycosylation, fatty acid acylation and subunit assembly.

To distinguish these possibilities, we have undertaken an analysis of the structures and functional sites of acetylcholinesterase using protein chemistry and immunologic techniques. Monoclonal antibodies have been raised to both the hydrophobic and asymmetric forms of Torpedo enzyme. Most of the clones express antibodies directed to the catalytic subunits and show equal reactivity towards the two classes of enzymes. For some antibodies reactivity is dependent on maintenance of tertiary structure while others react equally well with the native and denatured enzyme and hence recognize a linear sequence in the catalytic subunit. Two monoclonal antibodies show selectivity for particular enzyme forms. One shows about a hundred-fold selectivity for the catalytic subunits of the hydrophobic dimer (5.6S) form over the catalytic subunits of the tail-containing form (17+13S). The second reacts selectively with the tail-containing form and is most likely directed to the collagen-like tail unit. Antibodies selective for individual acetylcholinesterase species appear useful for immunocytochemical localization of individual acetylcholinesterase forms.

Analysis of peptide maps and selected sequences of the catalytic subunits from the two enzymes reveal a close homology between the two forms. Nevertheless, certain peptides differ between the forms as evidenced by peptide maps and compositions of isolated peptides. Peptide separation by HPLC has enabled us to isolate the active center, candidate peripheral site and antigenic peptides. Finally, nucleotide probes have been made to some of the peptides which should prove suitable for cloning the gene from existing Torpedo libraries. This approach will prove complementary to analysis of primary structure and distinguishing the molecular basis of the enzyme's polymorphism. With the prospect of having the gene(s) encoding the various acetylcholinesterase species and antibodies available that are selective for individual enzyme forms, it should be possible to isolate the m-RNA's responsible for expression.

2. Antigenic and Structural Differences in the Catalytic Subunits of the Molecular Forms of Acetylcholinesterase.

A mixture of the hydrophobic dimer (5.6S) and the asymmetric, tail-containing (17+13)S forms of acetylcholinesterase from *Torpedo californica* was used to immunize mice, and spleen cells from these mice were employed to produce nine hybridoma lines secreting antibodies against acetylcholinesterase. Antibodies from one of the lines showed a 100-fold greater affinity for the 5.6S species when compared with the catalytic subunits of the (17+13)S species. This difference in specificity was retained following denaturation of the two acetylcholinesterase species. Another line produced antibody directed only to structural subunits of the (17+13)S species while the remaining seven antibodies exhibited nearly equivalent cross reactivity for all of the forms of acetylcholinesterase. Tryptic peptides were generated from the catalytic subunits of the 5.6S and tail-containing acetylcholinesterase species, and high-pressure liquid chromatography profiles show at least four distinct peptides in the catalytic subunits for each enzyme species. Some of these peptides exhibit retention times different from the identified glycopeptides. Thus, it is likely that the catalytic subunits of the two molecular forms of acetylcholinesterase differ in primary structure and sites of antigenicity.

3. Antibodies that Distinguish between Molecular Forms of AChE Localize Differently on Torpedo Electrocytes.

Distinct molecular forms of AChE have been isolated from *Torpedo* electric organs and characterized biochemically. These include an asymmetric "tailed" species (17S+13S) and a dimeric hydrophobic species (5.6S). Monoclonal antibodies (MCAb) were raised against *Torpedo* AChE and have been characterized for antigenic specificity. Most antibodies show a high degree of cross reactivity between the different molecular forms of AChE, but two demonstrate selectivity for only one form. MCAb 4F3 is specific for the asymmetric species (and is unreactive towards this form of the enzyme following collagenase or trypsin removal of the tail section). MCAb 4E7 exhibits 100-fold selectivity for the catalytic subunit of the 5.6S hydrophobic dimeric form of AChE. Using these MCAbs as probes, we were able to examine the distribution of the different forms of AChE in *Torpedo* electric organ by immunofluorescence and immunoelectron microscopy. MCAb 4E7 shows a diffuse distribution, staining within the synapse giving the appearance of labeling both the innervated and non-innervated surfaces with approximately equal density. These patterns were confirmed using immunoelectron microscopy. In contrast, polyclonal antibodies raised against the asymmetric form bind both asymmetric and hydrophobic forms with equal avidity *in vitro*. Although these polyclonal antibodies do not appear to distinguish between molecular forms *in vitro*, the antigenic sites they localize *in situ* are less uniformly distributed than those localized by the monoclonal antibody directed against the hydrophobic dimer only. Localization by both light microscopy and electron microscopy reveal the same pattern of staining.

4. Molecular Aspects of the Biosynthesis and Disposition of the Multiple Forms of Acetylcholinesterase.

In the Torpedo electric organ both the elongated species of acetylcholinesterase which is confined to the basal lamina and the hydrophobic, dimeric species which associates with plasma membranes are present in sufficient abundance to obtain comparative information on the primary structures of the catalytic subunits. Although high pressure liquid chromatography of the corresponding tryptic peptides reveals substantial homology between the two forms, at least two of the non-oligosaccharide-containing tryptic peptides appear unique to each enzyme. Chromatographic separation of the tryptic and cyanogen bromide peptides has allowed us to identify the active site peptide, the oligosaccharide-containing peptides and surface peptides of the peripheral anionic site which react with the photoaffinity label, azidopropidium. Monoclonal antibodies have been prepared to both forms of the enzyme and while the majority show nearly equivalent reactivity with both forms of the enzyme, one shows a 100-fold selectivity for the catalytic subunit of the hydrohobic, dimeric species. Thus structural studies and the immunologic reactivity suggest that the acetylcholinesterase forms arise as separate gene products or from distinct RNA translation products.

Publications:

1. Palmer Taylor, Shelley Camp, Mark Schumacher, Kathleen MacPhee, Susan S. Taylor, Mary Kay Gentry, and B. P. Doctor: Molecular and structural basis for the polymorphic forms of acetylcholinesterase. Abstracts of the Fourth Annual Chemical Defense Bioscience Review, 1984.

2. B. P. Doctor and Palmer Taylor: Antigenic and structural differences in the catalytic subunits of the molecular forms of acetylcholinesterase. Proceedings of the Second International Meeting on Cholinesterases, 1983.

3. L. B. Howland, M. H. Ellisman, P. Taylor, T. J. Deerinch, B. P. Doctor, and S. J. Camp: Antibodies that distinguish between molecular froms of AChE localize differently on Torpedo electrocytes. Abstracts of the Annual Meeting, American Neuroscience Society, 1983.

4. Palmer Taylor, Shelley Camp, Stephanie Lee, Gabriel Amitai, Susan S. Taylor, and B. P. Doctor. Proceedings of the Second International Meeting on Cholinesterases, 1983.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 303108	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	111 WWMN
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code) (U) Studies on the Interactions between Prostaglandins and Glucocorticoids to Improve Chemotherapeutic Measures for Radioprotection, Radiation Disease, and Shock

12. SUBJECT AREAS
0615 Pharmacology; 0618 Radiobiology; 0601 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	13
85	2.0	12

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Division of Experimental Therapeutics Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR HEIFFER, M H
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (301)-427-5393
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) NIELSEN, C g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Radioprotectants, (U) Prostaglandins, (U) Glucocorticoids, (U) Metabolism, (U) Lab Animals, (U) Rats

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The purposes of this project are to further the understanding of how current anti radiation drugs function and to develop some of the scientific information necessary to design improved drugs and treatment regimens for radioprotection. The focus of the effort will be on better understanding and control of the vasoactive prostaglandins the production of which is stimulated by radiation of animal tissue. The ultimate goal is to improve the pharmacological protection of military personnel faced with the threat of radiation from nuclear weapons employment. It is anticipated that this information will also be of value to other military medical needs such as the treatment of radiation disease and prevention of irreversible shock.

24. (U) The first phase of this work will be to examine the effects of current radioprotective drugs on prostaglandin synthesis and on the controls of that synthesis rendered by glucocorticoids. This will be conducted in cellular, subcellular, and whole animal preparations. The next phase will be to examine how the effects of radioprotectant drugs can be modified by supplementary drugs that affect prostaglandin synthesis, i.e., steroids, antisteroids, aspirin, and indomethacin. Finally, the information obtained from these studies will be tested in whole animals irradiated under controlled conditions.

25. (U) 83 10 - 84 09 Analytical techniques have been developed to assay the level of glucocorticoid receptors in subcellular preparations of rat liver. Using these techniques it has been discovered that WR 1065, a principal metabolite of the radioprotector WR 2721, can stabilize these receptors. This may be one mechanism of WR 2721 action. Studies are now underway to determine the implications of this for prostaglandin synthesis. For technical report see WRAIR Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 111 Studies on the Interactions between Prostaglandins and Glucocorticids to Improve Chemotherapeutic Measures for Radioprotection, Radiation Disease, and Shock

Investigators:
Principal: Melvin H. Heiffer, Ph.D.
Associate: MAJ C.J. Nielsen, Ph.D., CPT A. Schroeder, Ph.D., J. Karle, Ph.D.

1. Description.

The purposes of this project are to further the understanding of how current antiradiation drugs function and to develop some of the scientific information necessary to design improved drugs and treatment regimens for radioprotection, radiation disease, and shock.

2. Progress.

Analytical techniques have been developed to assay levels of glucocorticoid receptors in whole cells and subcellular preparations of rat liver. Using kinetic techniques it has been discovered that WR 1065, a principal metabolite of the radioprotector WR 2721, can stabilize and even reactivate these receptors. This may be one mechanism of WR 2721 action.

Studies are now underway to determine the implications of this for prostaglandin synthesis. Along this line of work, procedures have been developed to measure a variety of prostaglandins and other arachidonate metabolites in cell-free medium and cytosol using high pressure liquid chromatography. Under the incubation conditions utilized, it has been found that arachidonic acid is converted by isolated rat liver hepatocytes to an unknown metabolite that is excreted back into the medium in high concentration. This metabolite does not appear to have HPLC characteristics like those of any known metabolite.

3. Future Work.

Studies continue on the salutary effect of WR 1065 on the glucocorticoid receptor. Efforts are underway to determine if this same effect can be elicited from whole cells in vitro under culture conditions. The parent WR 2721 will also be examined before and after dephosphorylation. Work continues on the unknown arachidonic acid metabolite to determine its nature by chromatography and mass spectrometry.

4. Publications.

Wright, N. and C.J. Nielsen. Preservation of rat liver glucocorticoid receptors *in vitro* by WR 1065, metabolite of the radioprotector WR 2721. *Ninth Int'l.* Congress of Pharmacology (1984), Abstracts, p. 1843P.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION DA 303109	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX
9. LEVEL OF SUM A. WORK UNIT					

NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	112 WWIM
CONTRIBUTING				
CONTRIBUTING	None			

TITLE (Precede with Security Classification Code)
(U) Effects of Ionizing Radiation on Intestinal Motility and Flora

12. SUBJECT AREAS
0613 Microbiology 0616 Physiology 0618 Radiobiology

START DATE [illegible] 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

CONTRACT GRANT		18. RESOURCES ESTIMATE		
DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
CONTRACT GRANT NUMBER		84	1.2	8
TYPE	d. AMOUNT	85	1.2	35
KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of Medicine
ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, D.C. 20307-5100
NAME OF RESPONSIBLE INDIVIDUAL Top, FH Jr	c. NAME OF PRINCIPAL INVESTIGATOR Sjogren, RW
TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-3530
GENERAL USE FINA	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Dubois A; Boedeker EC;
MILITARY CIVILIAN APPLICATION H	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Andrews G; Sjogren MH.

KEYWORDS (Precede EACH with Security Classification Code) (U) lab animals; (U) monkeys; (U) radiation; (U) gastrointestinal motility; (U)hepatitis; (U) diarrhea

TECHNICAL OBJECTIVE 24 APPROACH 25 PROGRESS (Precede text of each with Security Classification Code)

23. (U) Ionizing radiation is a major non-conventional weapon and may result in disabling gastrointestinal disturbances including anorexia, nausea, vomiting, diarrhea, abdominal pain and cramps, hepatitis, enteric infection, sepsis, and death. Our objective is to describe the effects, mechanisms, and medical countermeasures of ionizing radiation on gastrointestinal and hepatic structure and function. There is military relevance in this research.

24. (U) An initial study in rhesus monkeys with cutaneous and implanted intestinal electrodes and strain guages will describe effects of a single, lethal exposure of ionizing radiation on intestinal motility, morphology, and flora and on hepatic morphology. Subsequent studies will assess mechanisms and pharmacologic modulation of these effects and possibly effects of repeated sublethal radiation exposures on these parameters and on intestinal immune function.

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25. (U) A non-invasive method of recording gastric motility from cutaneous electrodes has been established and shown to correlate with recordings from surgically implanted electrodes and strain guages as published in DDS 1984;29:565. Studies in unsedated, chair adapted monkeys suggest that post-operative disruption of intestinal motility is more transient than previously suspected and that normal motility returns coincident with resolution of ileus. Preliminary studies suggest initial events of acute radiation sickness include vomiting, delayed gastric emptying, and slowed gastric electrical activity. Changes in emptying and electrical activity are reversed and vomiting averted by administration of metoclopramide as published in Gastroenterology 1984;86:1152. Computer programs to fully interpret motility are under development. Additional studies of gastric and small bowel motility, flora, and intestinal and hepatic morphology are needed and ongoing and use of drugs in addition to metoclopramide under consideration. Technical report in WRAIR Ann Prog Rpt, 831001-840930.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE

Project: 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 112 Effects of Ionizing Radiation on Intestinal Motility and Flora

Investigators:

Principle: LTC Robert W. Sjogren, MC
Associate: Andre Dubois, MD, PhD
COL Edgar C. Boedeker, MC
1LT (P) Gerard Andrews, MSC
MAJ (P) Maria H. Sjogren, MC

PROBLEMS AND OBJECTIVES

Nuclear weapons (both tactical and strategic) comprise the major non-conventional weapons system. In battlefield situations, troop exposure to non-conventional weapons could include radiation alone or radiation in combination with conventional and chemical-biological warfare agents. The dosage of radiation may be lethal or sublethal and repeated. The medical effects of these weapons particularly in combination and the appropriate prophylactic and therapeutic measures for troops and casualities are unknown. The requirement for rapid troop mobility confounds the situation by reducing time for intelligence gathering (ie. enemy capability), for medical planning, and for troop preparation (ie. immunization, antidote administration) and by increasing troop succeptibility to native enteric diseases (ie traveller's diarrhea, Salmonella, Shigella). Development of simple, broadly applicable medical countermeasures to both naturally occurring illnesses and non-conventional weapons would significantly enhance the combat effectiveness and flexibility of a unit. Such broad spectrum medical defense, however, runs the increased risk of adverse interaction with other medical measures and with other weapons. Although frequent and serious (often lethal) derrangements of intestinal function due to radiation have been clinically noted since the 1950's the apparently important effects of radiation on intestinal motility, flora, and immunologic function are unknown and their interactions with medical conditions associated with rapid deployment and CBW agents not investigated.

Major consequences of acute radiation exposure are acute radiation sickness and suppression of immunologic function. The bulk of medical radiation research was performed in the 1950's and 1960's. These studies demonstrated that gastrointestinal manifestations of acute radiation exposure were disabling and often lethal. They included: anorexia, nausea, vomiting, abdominal pain and cramps, diarrhea, hemorrhage, tenesmus, ileus, fluid and electrolyte imbalance, malabsorption, hepatitis, and extensive enteric infection that often led to sepsis and death. The pathophysiologic basis and therapeutic/prophylactic implications of these observations are virtually unstudied. By their nature these derrangements may be amenible to existing prophylactic or therapeutic measures if more were known of their pathophysiology. Over the past decade significant methodologic and conceptual advances in the understanding of intestinal neuromuscular and immune function have been made, but not applied to the problem of acute radiation sickness or its potential interface with native infection and with CBW agents.

In an ongoing study at AFRRI (Work Unit #WJ0059, "Effect of Irradiation and Antiemetics on Gastrointestinal Function"), Dr. Andre Dubois has recently demonstrated that 8 Gy of cobalt-60 whole body irradiation to rhesus monkeys resulted in an acute radiation sickness including vomiting, delayed gastric emptying, diarrhea, and extensive intestinal infection. Death due to infection or bone marrow suppression occurred after two weeks. Enteric infection has been severe. At autopsy, intestinal and hepatic tissue is obtained but not analyzed. This collaboration interprets additional data in this area from animals already undergoing study.

The objective of this work unit is to describe the effects of acute ionizing radiation and potential therapeutic agents on intestinal motility, morphology, and flora and on hepatic morphology in rhesus monkeys.

PROGRESS

1. Animal Model: Exploiting the large gastric muscle mass, methods have been developed to record gastric motility from ECG electrodes placed on the skin overlying the stomach of unsedated, chair adapted monkeys (electrogastrogram or EGG). This methodology is undergoing testing in humans as a non-invasive method of measuring gastric motility. Modification of standard surgically implanted serosal electrodes and strain guages to reduce size, tissue reaction, and tissue impedence have been completed and successfully used in these chair adapted monkeys. Cross correlation analysis of preliminary studies show excellent correlation between the three methods of assessing gastric motility (EGG, serosal electrodes, serosal strain guages), have been presented to the biennial meeting of the American Motility Society in July 1984 and published in a peer review journal.

To date, 7 monkeys have been implanted. Implantation of

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multiple gastric and intestinal electrodes and strain guages initially caused problems and 2 of the first 5 monkeys died postoperatively of apparent intestinal obstruction. Reduction in electrode numbers with omission of colonic electrodes has resolved the problem.

2. Computer Programs: Computer programs have been developed to appropriately filter and analyze slow electrical activity (slow waves) as recorded by the surface electrogastrogram and serosal electrodes and to perform cross correlation analyses. This approach has given complete analysis of gastric motility where muscle contraction is predominantly controlled by slow waves, but has been only partially successful in analysis of intestinal motility where motor activity is controlled by both slow waves and by fast electrical activity (action potentials or spikes). Existing computer programs designed to interpret spike activity accurately detected spikes, but did not fully interpret all spike patterns. Second generation programs are under development and should be completed within 4-8 months to interpret these spike patterns. In the interim, studies are being partially computer analyzed and partially manually analyzed for intestinal motility, and they are being archived on analog tape for eventual full computer interpretation.

3. Motility Effects: Monkeys display fasted (migrating motility complex) and fed (phase 2) motility patterns in the stomach and small intestine similar to man. Post operative disruption of the fasted pattern is brief and the migrating motility complex returns more rapidly than previously appreciated (usually within 1-2 days). Feeding is a major control mechanism.

Preliminary results in 4 monkeys suggest that 8 Gy cobalt-60 radiation and the consequent vomiting completely disrupt normal gastric and small intestinal motility resulting in abolition of migrating myoelectric complexes and reduction of slow wave frequency. Within 1 minute post radiation, gastric slow wave frequency increased from 3.4 cycles per minute (cpm) to 4.0 cpm, and then progressively decreased to 2.8 cpm within the next 5 minutes. During the subsequent 3 hours gastric slow wave frequency was highly variable with a dominant frequency of 2.6 cpm alternating with bursts of 5-6 cpm activity. Compensatory pauses followed the 5-6 cpm activity before the 2.6 cpm dominant frequency resumed. 2 of 4 monkeys received the antiemetic metoclopramide immediately prior to irradiation. Metoclopramide prevented radiation induced vomiting. The metoclopramide treated monkeys also showed an increase in gastric slow wave frequency within 1 minute post radiation. However, unlike controls, the increased slow wave frequency persisted during the subsequent 40-

50 minutes suggesting that metoclopramide may exert an antiemetic effect by alteration of gastric slow waves. Radiation induced vomiting and delay in gastric emptying were obsesrved only when gastric electrical activity was slowed. Metoclopramide did not effect intestinal slow wave activity. These results were presented at the third biennial meeting of the American Motility Society and published in a peer review journal.

4. Microbiologic Effects: Pre-irradiation jejuneal and ileal colony counts of aerobes, facultative anaerobes, and strict anaerobes have been extremely low (10^2 - 10^4 CFU/ml), but consistently higher in the ileum. Because of these low counts, assessment of the adequacy of the transport media used to transfer organisms from AFRRI to WRAIR is in progress. Unfortunately all 4 post radiation autopsies have been conducted several hours after death so that post radiation cultural data has not been meaningful. Greater familiarity with the time course of radiation sickness and closer collaboration with the veterinarians at AFRRI should correct this defect.

5. Morphologic Effects: All pre-irradiation liver biopsies are normal to light and electron microscopy. Post radiation liver biopsies are also normal to light microscopy, but autolysis has prevented adequate electron microscopic study. Because the early changes of radiation hepatitis may only be noted on electron microscopy, more timely autopsies are required. None of the 4 radiated monkeys developed clinical liver disease.

FUTURE PLANS AND RECOMMENDATIONS

1. Animal Model: Inclusion of colonic electrodes and strain guages is planned. Data is being examined to determine whether fewer gastric and/or small bowel electrodes can be used without significant data loss in order to accommodate the colonic electrodes If not, seperate studies using small bowel and colonic electrodes may be necessary.

2. Computer Programs: The computer programs mentioned in the "Progress" section along with appropriate graphic and statistical analyses should be completed during the next year. Studies archived on analog tape will be reanalyzed upon completion of these programs.

3. Motility Effects: Completion of initiated studies of placebo and metoclopramide treated monkeys will require a total of about 10 monkeys in each group. Manual analysis of small intestinal motility changes is underway, and will be greatly facilitated and enhanced by completion of computer programs. Studies of colonic

motility are planned. Based on results of this study additional studies involving radiation dose-response curves, effects of single and repeated sublethal doses of radiation, examination of other potential prophylactic and/or therapeutic measures (such as domperidone, cholinergic agents, WR-2721), and interactions of radiation effects with agents being evaluated for chemical defense (such as pyridostigmine, atropine, sarin) may be considered.

4. Microbiologic Effects: Assessment and probable revision of transport media and post-radiation autopsy collection proceedures are underway. Quantitative colony counts from the proximal and distal small intestine will be examined for total aerobes, facultative anaerobes, and strict anaerobes. If, as is expected, significant bacterial overgrowth is encountered post-radiation, it will be correlated with motility change and with metoclopramide treatment. In addition, simple characterization of the bacterial group most effected will begin (for example gram stain and culture on several selective media of aerobes). In addition, luminal contents will be screened for enterotoxin by Hela cell assay or rabbit ileal loop if toxin producing organisms are a reasonable possibility. Eventual studies of radiation effects on intestinal immune function, on the proliferation and invasion of microorganisms not native to the normal intestinal tract, and on the response to immunization and antibiotics may be considered.

5. Morphologic Effects: Additional histologic material (especially for electron microscopy) is essential. Revised autopsy proceedures are being implemented. In addition to hepatic histology, intestinal histology will be assessed and related to dose and time of radiation, effect on motility, extent of bacterial overgrowth, and clinical status.

ABSTRACTS AND PRESENTATIONS

1. Laporte JL, O'Connell L, Durakovic A, Sjogren R, Conklin JJ, Dubois A: Radiation induced alteration of gastric electrical activity in primates. Gastroenterology 1984; 86: 1152.

2. Laporte JL, O'Connell L, Durakovic A, Sjogren R, Conklin JJ, Dubois A: Cross correlation analysis of gastric motility following exposure to ionizing radiation in primates. Dig Dis Sci 1984; 29: 565. Presented at the Third Biennial Meeting of the American Motility Society, 22-25 July 1984, Milwaukee, WI.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 303110	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM
83 10 01	D. Change	U	U		CX	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	113 WWIN
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) Effect of Anticholinesterases on Gastrointestinal Motility

12. SUBJECT AREAS
0616 Physiology 0615 Pharmacology 0602 Bioengineering

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
[illegible] 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER				
c. TYPE	d. AMOUNT	84	0.5	52
e. KIND OF AWARD	f. CUM/TOTAL	85	0.5	35

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of Medicine
b. ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Sjogren, RW
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-3530
21. GENERAL USE FINA	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Van Albert, S
MILITARY/CIVILIAN APPLICATION H	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) lab animals; (U)rabbits; (U)rats; (U) signal analysis; (U)gastrointestinal motility; (U)cholinergic nervous system; (U)anticholinesterase

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Both chemical warfare agents and their medical countermeasures are active against the cholinergic nervous system, and both may result in disabling gastrointestinal disturbances including anorexia, nausea, vomiting, abdominal pain, and diarrhea. Our objective is to describe the nervous control of gastrointestinal function and effects and interactions of potential chem defense treatments on these functions. This is military relevant research.

24. (U) An initial study will establish a model to record GI motility from unrestrained animals and will develop computer programs to interpret motility patterns. Subsequent study will describe motility patterns, investigate control mechanisms, and study effects/interactions of selected drugs and potential chem defense treatments on these patterns. Potentially, effects of motility on gut flora, immune function, and transport could be studied.

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25. (U) A model has been developed permitting prolonged recording of GI motility and intravascular infusion of unrestrained rabbits. Unlike restrained animal studies, these demonstrate motility patterns similar to man. Feeding is a major control mechanism. Computer programs allowing multichannel event detection are complete and pattern recognition programs under development. Development of methods for ventilatory support and monitoring of cardiovascular and intestinal intraluminal pressure responses in anethesized animals permitted correlation of intestinal myoelectric patterns to intraluminal pressure change while demonstrating systemic drug effect as published in DDS 1984;29:563. Preliminary cholinergic studies in this model suggest the myenteric plexus exerts chronic inhibitory tone on motility, perhaps involving prostaglandins. Bethanechol and edrophonium are powerful cholinergic stimulants of motor activity. Scopolamine blocks effects. The level of these effects, their counterparts in unrestrained animals, and effects of chronic anticholinesterase administration are under investigation. Technical report in WRAIR Annual Prog Report, 831001-840930.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE

Project: 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 113 Effect of Anticholinesterases on Gastrointestinal Motility

Investigators:

Principle: LTC Robert W. Sjogren, MC
Associate: Mr. Stephen Van Albert, GS-13

PROBLEMS AND OBJECTIVES

The appropriate prophylactic and therapeutic measures to be employed for troops and casualities exposed or likely to be exposed to chemical warfare agents is of great concern to the Army Medical Command. This has recently been emphasized by the documented use of nerve gases in the Iraqi-Iranian conflict and the lingering questions of mycotoxin and nerve gas use in Thailand and Afghanistan. Typically these agents are active against the central and peripheral nervous systems. Their major mechanism of action is inhibition of acetylcholinesterase. At present, large doses of atropine (antimuscarinic agent) and pralidoxime (cholinesterase reactivator) are used in treatment and pyridostigmine (reversible anticholinesterase) is being investigated as a potential prophylactic agent to be used by troops likely to be exposed to chemical warfare agents.

Both chemical warfare agents and their medical countermeasures (treatment or prophylaxis) have many cholinergic and anticholinergic effects. Although the importance of cholinergic innervation to the gastrointestinal function is well established and interference with its function (as by pyridostigmine) may result in disabling nausea, vomiting, diarrhea, abdominal cramps, malabsorption, and incontinence, the specific physiologic mechanisms and pathways by which cholinergic stimuli manifest their effects are unclear These effects have been noted in monkeys receiving anticholinesterase agents (such as pyridostigmine) and may limit their usefulness as potential prophylactic agents. In addition, these agents have significant interactions with commonly used drugs. Such drugs might be required by command edict (i.e. the antimalarials), used illicity (i.e. marijuana, cocaine, amphetamines, heroin), medically prescribed (i.e. opiates, sedatives, tranquilizers, anti-inflammatory agents, antibiotics) or normally consumed (i.e. caffeine, nicotine, alcohol).

LABLE

Recent development of drugs highly specific to cholinergic receptor subtypes (eg. DMPP, nicotinic agonist; pirenzepine, M-1 antagonist; 4-DAMP, M-2 antagonist) and the encouraging reports of efforts to clone the acetylcholine receptor have greatly expanded specificity of investigational probes and potential therapeutic options. They have also suggested that studies on cholinergic mechanisms in the gastrointestinal tract may be important to military relevant disorders not related to chemical warfare (ie. peptic ulcer, gastroesophageal reflux, diarrhea, intestinal water and electrolyte transport, and functional bowel syndrome).

Gastrointestinal motility plays a critical role in permitting normal nutrient ingestion and elimination, in allowing for normal absorption of nutrients, medications, fluid and electrolytes, in maintaining a proper ecologic balance and preventing enteric infection, and in maintaining proper immunity. In addition, disordered gastrointestinal motility may result in disabling anorexia, nausea, vomiting, diarrhea, constipation, abdominal pain, and cramps. Since the dominant innervation of the gastrointestinal tract is cholinergic, one would expect it to be an important factor in the control of gastrointestinal motility. Although cholinergic factors exert major effects on gastrointestinal motility, the physiologic control mechanisms are extremely complex and involve significant cholinergic interactions with non-cholinergic mechanisms (including adrenergic pathways, other neuropeptides, dynorphins and opiates, prostaglandins, and cyclic nucleotides). Clinical examples of disordered motility have been documented in certain enteric infections, bacterial overgrowth syndromes, functional bowel syndrome, and intestinal pseudoobstruction and gastrointestinal side effects of cholinergic and anticholinergic drugs are frequent.

For several years, our laboratory has studied the effects of bacterial enterotoxins on gastrointestinal motility in rabbits. Many of the methods used in these studies are potentially adaptable to study cholinergic effects on gastrointestinal motility. These studies have developed first generation computer programs which assist interpretation of gastrointestinal motility and have investigated the effects of bacterial enterotoxins on motility in anesthesized animals. The computer programs fail to fully characterize important motility events and do not relate the two major motility patterns recorded (spikes and slow waves) to each other - an important relationship in the control of intestinal motor function. In a project on experimental colitis in rabbits (M10-81), difficulties were encountered in establishing a model for recording motility from chronically implanted electrodes in unanesthesized rabbits. The difficulties were primarily technical relating to type of electrode used and the requirement to restrain the animal during recording. We have proposed a new electrode better tolerated by the animal and a swivett assembly to allow recording from unrestrained, unsedated animals as being a more physiologic preparation. These developments, however, have not been further pursued.

The specific objectives of this work unit are:

1. To establish a model to record gastrointestinal motility from unrestrained, unsedated rats and rabbits.
2. To define the normal fasted and fed motility patterns.
3. To establish second generation computer programs that detect, characterize, and interrelate the motility patterns.
4. To define the cholinergic controls of normal fasted and fed motility, the effects of cholinergic agents on motility (especially organophosphorous compounds and pyridostigmine), and the effects of potentially interactive drugs (such as delta-9-tetrahydrocannibinol, cocaine, and the antimalarials).

PROGRESS

Progress to date suggests that methods can readily be developed to study the gastrointestinal effects of cholinergic agents and that these effects are important.

1. Chronic Animal Model; Fasted and Fed Motility: A model to record gastrointestinal motility from rabbits with the newly designed, chronically implanted electrodes and strain guages has been developed. Development of methods for electrode fabrication and surgical implantation was rapid and 5 of the last 6 rabbits so implanted are clinically healthy with normal bowel function and weight gain more than 2 months following surgery. Motility recordings during restraint from these animals have been technically satisfactory. Specialized metabolic cages with swivett assemblies have been constructed and appropriate recorder upgrades are in progress to allow continuous 24 hour recording and intravascular infusion of unrestrained, unsedated animals. Construction of the swivett assemblies was unexpectedly slow, but is now complete and animal studies using the equipment initiated in August 1984. Preliminary results on 2 rabbits suggest that rabbits demonstrate fasted (migrating motility complex) and fed (phase 2 activity) motility patterns similar to primates. These patterns were not previously noted in recordings of restrained or anesthesized rabbits and emphasize the critical importance of using a physiologic model.

2. Computer Programs: Development of second generation computer programs was initially slow because of delay in hardware acquisition which is now nearly complete. Programs have been

developed on a dedicated minicomputer (Hewlett Packard A700) to allow continuous multichannel data collection, digital filtering, slow wave frequency analysis (subthreshold variations of muscle membrane polarization that control the enviornment in which action potentials may occur), spike burst detection (contraction producing action potentials), and timing of spike bursts in relation to the controlling slow wave frequency. These programs are presently completing confidence testing. Programs remaining to be developed are technically less complex and include those designed to characterize spike burst frequency and power (strength of peristaltic contraction), transfer summarized data from the H-P A700 to the DEC VAX 11/780 computer (an Institute wide resource), interpret spike burst patterns (on the VAX), and determine spike burst propagation (on the VAX). Completion of these programs should require 4-8 months and should allow complete analysis of motility recordings from all animal models. Incomplete, but usable data should be available from the first section of these programs within 2 months.

3. Neurotransmitter Studies: During the period of development of the chronic animal model and second generation computer programs, effects of cholinergic agents on gastrointestinal motility was studied by extending established techniques using an anesthesized rabbit model and existing first generation computer programs. Although, as previously suggested, this model is less physiologic than an unrestrained model, it permits experimental controls that the unrestrained model does not. Hence, the two models must be used together. Techinques of tracheostomy and ventilatory support (during experiments with tetrodotoxin); selective intraarterial drug injection; continuous monitoring of cardiovascular and respiratory responses to drugs (to demonstrate and monitor systemic effects); and simultaneous measurement of intraluminal intestinal pressure changes, muscle electrical events, and intestinal fluid production have been developed.

Results showing that intestinal myoelectric events and patterns could be correlated with intraluminal pressure changes and with fluid accumulation were presented in July 1984 to the biennial meeting of the American Motility Society and published in a peer review journal. Preliminary results of drug studies have shown that tetrodotoxin (neural blocker) and indomethacin (prostaglandin synthetase inhibitor) both stimulate short, high amplitude, frequent bursts of intestinal myoelectric and contractile activity. Bethanechol (muscarinic agonist) and edrophonium (anticholinesterase) elicit prolonged bursts of intestinal motor activity reminiscent of diarrheal patterns evoked by shigella and cholera enterotoxins, but of greater duration, amplitude, and frequency. All of these events are blocked by scopolamine (muscarinic antagonist). The colon appears much more sensitive to these effects than either the stomach or small intestine. Variation in sensitivity to drug effects between rabbits (up to 100 fold dose variation) and alteration in response to repeated doses (presumably cumulative drug effect, receptor alteration, or tachyphylaxis) have made establishment of dose-response curves difficult. We are presently pursuing the possiblilty that initial animal calibration using sub- and suprathreshold doses of bethanechol can be used to place the animal on the proper dose-response curve for multiple cholinergic agents.

Interpretation of these preliminary results is difficult and uncertain. It would appear that intestinal muscle is under chronic inhibitory neural tone (presumably from the myenteric nerve plexus) and that this inhibition may involve prostaglandins. Muscle contraction may result from intermittent lifting of this tonic inhibition. Cholinergic agonists are powerful stimulants of intestinal motor activity and may act by removing tonic inhibition of the myenteric plexus, directly stimulating lower motor neurons, and/or directly stimulating smooth muscle receptors.

FUTURE PLANS AND RECOMMENDATIONS

Based on the first year's success, definition of a full project line by FY 86 or FY 87 seems likely. The purpose of continuing the ILIR for 1-2 more years will be to complete methodologies under development and to define broad areas of research importance in cholinergic control of gastrointestinal function. This will assure directed research using established methods.

1. Chronic Animal Model; Fasted and Fed Motility: Full characterization of normal fasted and fed gastric, small bowel, and colonic motility in unrestrained, unsedated rabbits will performed. A similar model in rats will be developed (electrodes, cages, swivetts, etc. are designed to fit either species) and herbivore motility patterns will be compared to those of rodents. Although rats are more technically difficult to implant with electrodes than rabbits, they may prove to be a better animal model because they are less expensive and they consume a diet more like that of man.

2. Computer Programs: The computer programs mentioned in the "Progress" section along with appropriate graphic and statistical analyses should be completed during the next year.

These will utilize both the Hewlett Packard A700 and the DEC VAX 11/780 computers. Studies performed prior to completion of these programs will be stored on analog tape and reanalyzed upon program completion.

3. Neurotransmitter Studies: Further definition of cholinergic responses and the perplexing chronic inhibitory activity exerted by the myenteric plexus involving prostaglandins will be undertaken. The first step will be to standardize the cholinergic responsiveness of each study animal perhaps by placement on a dose-response curve using test doses of bethanechol. Secondly, using the acute, anesthesized rabbit model activity of nicotinic and muscarinic receptors will be probed using: DMPP (nicotinic agonist), a-bungarotoxin (nicotinic blocker), bethanechol (muscarinic agonist), pirenzepine (M-1 blocker), and 4-DAMP (M-2 blocker). Response variation in different anatomic regions will be noted. The level of action of these agents (myenteric plexus, lower motor neuron, direct smooth muscle) will be examined by noting their effect in the presence of hexamethonium (ganglionic blocker), tetrodotoxin (neural blocker), and botulinum toxin (blocks acetylcholine release). The role of prostaglandins will be investigated using infusions of prostaglandin E_1 and prostaglandin F_{2a} and the combination of indomethacin with hexamethonium and tetrodotoxin. Subsequent evaluation of the prostaglandin response will involve use of indomethacin and specific prostaglandins with specific neural agonists and antagonists.

Finally, the effects of neurotransmitters (particularly acute and chronic administration of pyridostigmine and diisopropylfluorophosphate) will be examined on the fasted and fed motility of unrestrained, unsedated animals. Route and timing of administration (particularly with regards to meals) and subsequent effect on motility will be examined. Coadministration of other cholinergic and anticholinergic agents and potentially interactive drugs (such as delta-9-tetrahydrocannibinol, cocaine, and the antimalarials) will be examined. Full characterization of significant motility responses may be probed as described in the anesthesized rabbit model.

ABSTRACTS AND PRESENTATIONS

1. Sjogren RW, Saenz JF, Wardlow M: Relationship of Myoelectric Activity Patterns to Intraluminal Pressure Change in Acute in vivo Rabbit Ileal Loops. Dig Dis Sci 1984; 29: 563. Presented at the Third Biennial Meeting of the American Motility Society, 22-25 July 1984, Milwaukee, WI.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 303112
2. DATE OF SUMMARY: 84 10 01
RT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	114 WWSA
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code): (U) Antigenic Epitopes of Dengue Viruses

12. SUBJECT AREAS: 0613 Microbiology 0603 Biology

13. START DATE: 8310
14. ESTIMATED COMPLETION DATE: Cont
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT/GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	123
85	2.0	125

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code): Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL: TOP, F H JR
d. TELEPHONE NUMBER (include area code): (202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME: Division of Communicable Diseases & Immunology
b. ADDRESS:
Walter Reed Army Institute of Research
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR: FEIGHNY, R J
d. TELEPHONE NUMBER (include area code): (202)-576-3478
f. NAME OF ASSOCIATE INVESTIGATOR (if available): BURKE, D S
g. NAME OF ASSOCIATE INVESTIGATOR (if available): BRANDT, W E

21. GENERAL USE: FINA
MILITARY CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Arbovirus; (U) Dengue virus; (U) Antigen; (U) Immunology

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To characterize the important antigenic epitopes of dengue viruses by isolation and purification of the envelope glycoprotein. This is an essential preliminary step to the development of alternative vaccines to the attenuated, live virus vaccines. Vaccines are the only feasible means for preventing epidemic dengue fever in American soldiers.

24. (U) Dengue viral envelope proteins will be purified in natural and denatured forms from large volume cell culture harvests. Purification will utilize high speed centrifugation, isoelectric focusing and affinity chromatography. Epitopes will be distinguished by their reactivity with monoclonal antibodies and characterized by amino acid sequencing.

25. (U) 83 10 - 84 09. Dengue 2 viral envelope glycoproteins have been purified in native forms which react with monoclonal antibodies. Amino acid sequencing is being undertaken and the epitopic regions are being characterized. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 114 Antigenic Epitopes of Dengue Viruses

Investigators:

Principal: Robert J. Feighny, Ph.D.
Walter E. Brandt, Ph.D.
COL William H. Bancroft, M.D.

Associates: Mrs. Jeanne Burrous, M.S.
SP5 Julius Anongos

Problems and Objectives

Development of live attenuated dengue virus vaccines is hindered by the low titer and low infectivity of some attenuated viruses. The best alternative approach is the preparation of synthetic polypeptide vaccines incorporating polypeptides capable of stimulating a neutralizing antibody response and representing the principle type-specific epitopes. USAMRDC contractors are already cloning the dengue virus genomes. One additional step is required, the preparation of large amounts of purified viral envelope glycoprotein.

Progress

Sufficient dengue-2 viral envelope glycoprotein has been purified in both nature and denatured forms to permit sequencing. The protein reacts with monoclonal antibodies prepared against the viral neutralizing antigen by radioimmune assays. By polyacrylamide gel electrophoresis, the polypeptide appears as the major band with a molecular weight of 59,000 daltons. In collaboration with an outside contractor, cDNA has been prepared and cloned by recombinant DNA technology into *E. coli*. The cloned material has been shown to the dengue-2 type specific. This cloned nucleic acid is now being inserted into an expression vector in order to produce proteins which will be characterized by monoclonal antibodies. The objectives of the first year have been surpassed.

Recommendation

Future efforts should be directed to the amino acid and cDNA sequencing of the protein and viral genome, and polypeptides produced in expression vectors should be characterized. The methodology developed in the work on dengue-2 should be applied to the other three serotype dengue viruses.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION DA 303113	2. DATE OF SUMMARY 84 10 01	*REPORT CONTROL SYMBOL* *DD-DR&E(AR) 636*
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX
9. LEVEL OF SUM A. WORK UNIT					

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	115 WWSB
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE *(Precede with Security Classification Code)*
(U) Immunopotentiation of Microbial Peptide Antigens

12. SUBJECT AREAS
0613 Microbiology 0703 Organic Chemistry

13. START DATE: 83 10
14. ESTIMATED COMPLETION DATE: Cont
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	2.0	35
85	2.0	65

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)*
Washington, DC, 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
Top, F H Jr
d. TELEPHONE NUMBER *(include area code)*
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME
Division of CD&I
b. ADDRESS
Walter Reed Army Institute of Research
Washington, DC, 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
Lowell, G H
d. TELEPHONE NUMBER *(include area code)*
(202)-576-3058
f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
Seid, R
g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
Zollinger, W

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Peptide Vaccine; (U) Adjuvants; (U)Outer Membrane Proteins (U) Trypanosomes; (U) Mice; (U) MDP

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Pure peptide vaccines need non-toxic adjuvants and carriers to protect against many militarily important diseases. Our purpose is to develop safe and efficacious immunopotentiating methodology and agents which will enhance the immunogenicity of peptide vaccines. Trypanosomes cause extensive disease among millions of people in Africa. Troops deployed in endemic areas expect high morbidity. Current treatment is insufficient or non-existent. Synthetic vaccines identical to conserved peptides of the variable surface glycoprotein of african trypanosomes are used in this study.

24. (U) Mice are immunized with conserved trypanosome peptides synthesized by Peninsula Labs with and without hydrophobic amino acid or fatty acid feet. Peptide immunization using adjuvant methodology with human vaccine potential include auto-micelle formation, hydrophobic complexing to meningococcal outer membrane protein vesicles and alum. These are compared to potential or known toxic adjuvants and carriers including Freund's adjuvant, muramyl dipeptide and KLH.

25. (U) 83 10 - 84 09 Experimental vaccines, given with or without alum, contained peptides made with hydrophobic feet which were either auto-complexed into micelles or complexed (by 3 different methods) to vesicular or fragmented outer membrane proteins. Control vaccines, given with or without Freund's adjuvant, consisted of peptides alone or covalently complexed to KLH. Preliminary data from sera of hundredes of mice indicate that anti-peptide IgG and IgM can be induced by these vaccines. Problems included delayed aquistion of peptides and development of optimal and efficient complexing methodologies. For technical report see WRAIR Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 1498, 83 MAR — EDITION OF MAR 68 IS OBSOLETE.

Project 3A161101A91C In-House Laboratory Independent Research

Work Unit 115: Immunopotentiation of Microbial Peptide Antigens

Investigators

Principal: LTC George H. Lowell, M.D.

Associates: Lynette F. Smith, M.S.
Wendell D. Zollinger, Ph.D.
Robert C. Seid, Jr., Ph.D.

Objectives

The objective is to develop safe and efficacious immunopotentiating methodology and agents which will enhance the immunogenicity of peptide vaccines. The synthetic peptides used in this study are prime vaccine candidates since they are identical to conserved portions of the variable surface glycoprotein of African trypanosomes and are common to many trypanosome variants.

Progress

We have previously shown that meningococcal outer membrane protein (OMP) enhances the immunogenicity of hypo-immunogenic polysaccharides, LPS or proteins when hydrophobically complexed to these antigens. Since OMP has been safely administered to thousands of people, we have concentrated on comparing the adjuvanticity of OMP to that of KLH (a typical protein carrier) and Freund's adjuvant which is too toxic for human use.

OMP was extracted and partially purified in vesicular and fragmented forms. Since the vesicular form has been used most extensively in previous experiments and in human trials, this preparation was tested first although the fragmented form is more free of LPS. Over the last six months, peptides corresponding to two conserved regions of the variable surface glycoprotein of trypanosomes were synthesized according to our specifications by Peninsula Labs. In order to complex the peptides to OMP in a way which leaves their hydrophilic antigenic determinants available for recognition by antigen processing cells, a fatty acid or amino acid hydrophobic 'foot' was covalently added to one end of the peptide. Two methods previously used to complex antigens to OMP (ethanol co-precipitation and immunobead treatment) were found to be inappropriate for peptide complexing; two other methods were developed:

Sonication of the OMP and peptide mixture in water followed by lyophilization or mixing of OMP and peptide in the presence of detergent followed by dialysis across a 1000 MWCO membrane to retain the peptide so that its hydrophobic 'foot' complexes with OMP as the detergent is gradually dialyzed away. Ultracentrifugation was used to separate the peptide-OMP complexes from peptide which remained free or auto-complexed into peptide micelles.

Mice were immunized and boosted with auto-complexed and OMP-complexed peptides with and without alum; controls consisted of KLH-conjugated peptide or peptide alone with or without Freund's adjuvant or alum. Sera, collected twice before and twice after booster immunizations, were assayed using an ELISA with either free peptide of BSA-conjugated peptide as the detecting antigen.

Results from many of the sera are in progress. Preliminary data clearly indicate that addition of a hydrophobic foot enhances the immunogenicity of a peptide even in the absence of other adjuvants. Furthermore, the data suggest that complexing peptide to OMP via the dialysis method and immunizing with alum may be more immunogenic than peptide covalently conjugated to KLH and given with Freund's adjuvant. Confirmation of this data would imply that a method for designing a safe and immunogenic peptide vaccine that is simple, practical and inexpensive to produce may be readily available.

Future Plans:

1. Confirmation of ability of hydrophobic foot and/or OMP and alum to enhance the immunogenicity of trypanosome peptides with particular attention to:

a. Dialysis methodology - the detergent, amount of OMP.

b. comparison of amino acid and fatty acid 'feet' with respect to size, composition and attachment site.

c. Comparison of vesicular and fragmented forms of OMP with low or negligible levels of LPS.

d. Nature of peptides that can be immunopotentiated - required number and charge (+ , - or neutral) of amino acids, cyclized vs. 'free' peptides, etc.

2. Ability of peptide vaccines to induce antibodies that bind to and/or neutralize autologous and cross-reacting strains of trypanosomes and their native proteins in order to develop a safe and efficacious anti-trypanosome vaccine.

3. Comparison of Hydrophobic foot-OMP-alum adjuvanticity to that of muramyl dipeptide (MDP), steroyl-MDP, STM (E. coli outer membrane B-cell mitogen) and liposomes.

4. Application of hydrophobic foot-OMP methodology to other militarily relevant peptides (e.g. malaria).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 303114	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D.Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	116 WWSC
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) DNA Hybridization Identification of Leishmania in Mammals and Insect Vectors

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE 83 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	1.0	5
c. TYPE	d. AMOUNT			
e. KIND OF AWARD	f. CUM/TOTAL	85	1.0	10

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Inst of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Division CD&I Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Hockmeyer, W T
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-3544
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Jackson, P g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) DNA Hybridization; (U) Leishmania; (U) Parasite; (U) Identification

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objective of this work is to develop a sensitive DNA Hybridization procedure to detect and identify Leishmania parasites. Leishmaniasis is endemic in Africa, the Mid East, Indian Subcontinent and South America posing a significant potential threat to military operations in these areas.

24 (U) DNA will be extracted from Leishmania of different species and labelled by radioactive and non-radioactive means to construct species-specific DNA probes. These probes will be hybridized to Leishmania from infected tissues or insect vectors to develop sensitive methods for detecting and identifying Leishmania. Sensitive detection methods will assist Leishmania chemotherapy procedures, drug and vaccine development and epidemiology studies.

25 (U) 83 10 - 84 09 DNA hybridizations with 32 P-labelled kDNA networks revealed that several Leishmania species (L. tropica, L. donovani, L. donovani infantum) share nucleotide sequences so intact networks are poor identification probes for these species. However, L. mexicana, L. donovani, and L. aethiopica kDNA networks can be used as hybridization probes for these species. Fragments of kDNA from L. donovani, L. tropica, and L. donovani infantum cloned into E. coli plasmids hybridized in a species-specific manner to parasites in human or animal biopsy material or insect vectors. As few as 1000 Leishmania promastigotes can be detected with 32P or biotin-labelled kDNA networks. Results indicate that DNA hybridization is a sensitive and relatively rapid means to detect and identify Leishmania. We are extending the research to obtain species specific clonded fragments of kDNA from New World Leishmania and to increase the sensitivity of the procedure. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 1983 - 30 Sep 1984.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

PROJECT 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 116 DNA Hybridization Identification of Leishmania in Mammals and Insect Vectors

Investigators:
Principals: Peter R. Jackson, Ph.D.
LTC Wayne T. Hockmeyer, MSC

Associates: John A. Wohlhieter, Ph.D.
Mr. John M. Stiteler

Problems and Objectives:

The problem under study is the development of vaccines against human parasites that cause leishmaniasis, trypanosomiasis, and malaria. These diseases impede the military performance of troops deployed in endemic areas, necessitating effective vaccination programs. Parasite identification is crucial to vaccine production and the current goal is to develop *Leishmania* identification methods based on kinetoplast DNA (kDNA) analysis. In addition to objectives detailed in the last annual report, new objectives are to determine the following points: 1) The extent of the species specificity and organism detection sensitivity of fragments of kDNA from *L. donovani*, *L. donovani infantum*, and *L. tropica*, cloned into *E. coli* plasmids, using DNA hybridization procedures. 2) If, in collaboration with Codon, Inc., (Brisbane, CA), specific kDNA fragments from *L. mexicana mexicana*, *L. mexicana amazonensis*, *L. braziliensis guyanensis*, *L. b. panamensis* and *L. b. braziliensis* can be cloned into *E. coli* to produce DNA probes for these New World parasites. 3) If the sensitivity and specificity of non-radioisotopically-labelled kDNA probes can be improved by adjusting conditions during hybridization. 4) If kDNA probes can be used to detect *Leishmania* in sandflies by hybridization. 5) If certain *Leishmania* from U.S. military personnel in Panama, and dogs in the U.S., that were characterized as *L. donovani*-like by isozyme (by Dr. Richard Kreutzer) or radiorespirometric (by Dr. Joan Jackson) procedures, are *L. donovani*-like using *L. donovani*-specific kDNA probes in DNA-hybridization tests. 6) If a simple, non-radioisotopic test can be conducted for detecting *Leishmania* in patient biopsy material using samples from patients at WRAMC and the WRAIR Unit in Nairobi, Kenya.

Progress:

In collaboration with Codon, Inc. we have developed clones of *E. coli* containing plasmids with kDNA fragments specific for *L. donovani* (six clones), *L. tropica* (two clones) and *L. donovani* infantum (two clones). By DNA hybridization the cloned kDNA detects as few as 1000 L. donovani, 10,000 L. donovani infantum and 100,000 L. tropica parasites in a species specific manner, by radioisotopic and non-radioisotopic methods. The DNA sequence of the probes has been determined. All are different from each other but within a particular kDNA fragment, repeated sequences occur and may confer species-specificity in hybridization reactions. Hybridizations conducted with pLD3, a L. donovani-specific kDNA clone, indicate that it binds to kDNA from Leishmania causing visceral disease in Africa, Europe, Brazil and Honduras and may be a universal DNA probe for visceralizing Leishmania. L. tropica - specific kDNA clones hybridize to parasite's from Russia, Israel and Africa and may be universal L. tropica markers. One L. donovani infantum probe hybridizes to kDNA from parasites isolated from a dog and a human in France. By restriction enzyme digest tests of the two parasites kDNA, we found that they were identical, proving that this strain of human L. donovani infantum is also in dogs. We have conducted extensive studies on L. donovani chagasi isolates from Brazil and Honduras. By restriction enzyme digestion of kDNA, we found that 14 Brazilian isolates (eight from the Bahia region and 6 from an island 1000 miles away) are identical and similar to a Honduras L. donovani chagasi. Another L. donovani chagasi from Honduras is unique. We also determined that some U.S. military personnel training in Panama acquired Leishmania with kDNA that has restriction enzyme-produced fragments very similar to those of L. donovani chagasi from Honduras. Hybridization studies with L. donovani-specific kDNA probes is being conducted to determine if Leishmania with the potential for causing visceral disease are in Panama. We have also developed a simple, rapid, fluorescence-microscope-based procedure for detecting viable intracellular and extracellular Leishmania. The procedure also detects viable pathogenic protozoa of many other genera, including Plasmodia and Trypanosoma.

Recommendations:

1. Contract research with Codon, Inc. should be continued for obtaining kDNA hybridization probes specific for all L. braziliensis and L. mexicana subspecies in the New World. In addition Codon will supply protocols for increasing specificity and sensitivity of non-radioisotopic DNA hybridization procedures using Leishmania specific kDNA probes.

2. File, with Codon, Inc., for patents on the use of Leishmania specific kDNA probes already developed.

3. Conduct extensive hybridization tests with New and Old World Leishmania kDNA probes to determine their specificity and sensi-

tivity and select those with the best potential for field use in *Leishmania* diagnosis.

4. Determine the optimal methods for radioisotopic and non-radioisotopic detection of *Leishmania* in human and animal biopsy material and in sandflies. Protocols are to be developed with WRAIR Entomology Department personnel, the Infectious Disease Unit at WRAMC and the WRAIR Unit in Nairobi to obtain material for this work.

5. Determine if *Leishmania* from U.S. Military personnel in Panama resemble visceralizing *Leishmania* using kDNA hybridization and restriction enzyme digestion methods.

6. Determine if kDNA probes can be used for evaluating the successful elimination of *Leishmania* from experimental animals used in research with new anti-*Leishmania* vaccine or drug trials. Protocols for the work will be developed with Department of Immunology and Parasitology personnel.

7. Expand ongoing restriction enzyme fragment study of kDNA from *Leishmania* causing visceral disease in Kenya and Brazil to determine the extent of *Leishmania* populations in endemic areas.

8. Determine if short repeating sequences in the cloned fragments of kDNA are responsible for species-specificity of the fragments in DNA hybridization tests.

Papers In Press or Submitted:

1. Jackson, P.R., J.A. Wohlhieter, J.E. Jackson, P. Sayles, C.L. Diggs, and W.T. Hockmeyer. 1984. Restriction endonuclease analysis of *Leishmania* kinetoplast DNA characterizes parasites responsible for visceral and cutaneous disease. Am. J. Trop. Med. Hyg. (In Press).

2. Lawrie, J.M., P.R. Jackson, J.M. Stiteler, and W.T. Hockmeyer. 1984. Identification of pathogenic *Leishmania* promastigotes by DNA-DNA hybridization with kinetoplast DNA cloned into *E. coli* plasmids. Am. J. Trop. Med. Hyg. (In Press).

3. Jackson, P.R., 1984. Differentiation of *Leishmania* Species and Strains by Analysis of Restriction Endonuclease-produced Kinetoplast DNA Fragments and DNA-DNA Hybridization with ^{32}P-kDNA From Type Isolates. In: UNAEC Workshop On Atomic Energy Applications in Parasitology. E. Hayunga and M. Steck (eds) (In Press).

4. Jackson, P.R., M.G. Pappas, B.D. Hansen. 1984. Fluorogenic substrate detection of viable intracellular and extracellular pathogenic protozoa. *Science* (In Press).

Published Abstracts:

1. Jackson, P.R., J.E. Jackson, M.G. Pappas, B.D. Hansen. 1984. Fluorogenic substrate detection of viable intracellular and extracellular pathogenic protozoa. Fed. Proc. 43:1630.

2. Lawrie, J.M., P.R. Jackson, J.M. Stiteler, and W.T. Hockmeyer. 1984. Pathogenic *Leishmania* promastigotes identified by hybridization with kinetoplast DNA cloned into *E. coli* plasmids. Fed. Proc. 43:1808.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	DA 303115	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	WWIY 117
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE *(Precede with Security Classification Code)*
(U) Lymphocyte Paralysis in Malaria - The Role of Cyclic Nucleotide Metabolism

12. SUBJECT AREAS
0615 Pharmacology, 0616 Physiology, 0603 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	Cont	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	3.0	40
85	3.0	40

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS Division of Medicine Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Wiesmann, W P
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-3636
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Webster, H K g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Malaria; (U) Lymphocytes; (U) Acquired Immun ficiency; (U) Cyclic Nucleotides; (U) Adenosine Receptor

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23.(U) 1. Define lymphocyte subset profiles in relation to the stage of infection and immun status of malaria infected individuals. 2. Define purine nucleotide pathways in normal and infected lymphocytes with emphasis on adenylate cyclase substrate availability. 3. Study lymphocyte adenylate cyclase in membrane preparations. a. Is an adenosine receptor interlock with activation? b. What is the status of the GTP coupler? 4. Quantitate the effects of pharmacologic manipulation of cAMP content with known phosphodiesterase inhibitors and immunostimulents of lymphocyte blastogenesis. There is military relevance in this research.

24.(U) Lymphocytes obtained from malaria patients will be identified with available immuno-fluorescent tags. Cell sorting for purification of subsets will be performed with the Coulter fluorescent activated cell sorter available at WRAIR. Extractions for nucleotides profiles, purine pathway analysis, and cAMP content will be measured on selected subsets utilizing techniques already developed. Adenylate cyclase extraction and analysis will be performed at WRAIR. The effects of various immunostimulents and cAMP analogues will be performed in functional assays currently available at AFRIMS.

25.(U) 83 10 - 84 - 09 cAMP generation is reduced 90% in lymphocytes from humans and monkeys infected with malaria but recovers in parallel with the restoration of blastogenesis after treatment. Exogenous cAMP restores the mitogen response in lymphocytes from malaria infected humans and monkeys suggesting a causal relationship. Analysis of the adenylate cyclase regulatory subunit has identified a reversible defect in the N_i site. Alterations in helper/suppressor surface markers may be mediated by this defect in cAMP production. Experiments a now in progress to evaluate the possible restoration of the immune response in malaria by pharmacological manipulation of lymphocyte cAMP production. (Presented to the Am. Fed of Cl Res, May, 1984, Science, submitted for publication). For technical report see Walter Reed A Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project: 3A161101A91C In-House Laboratory Independent Research

Work Unit: 117 Lymphocyte Paralysis in Malaria - The Role of Cyclic Nucleotide Metabolism

Investigators:

Principal: LTC William P. Wiesmann, MC
Associate: MAJ H. Kyle Webster, MSC

Problems and Objectives

The emergence of resistant strains of the human malaria parasite P. falciparum to recently introduced antimalarial drugs poses a tremendous threat to the health of troops deployed in endemic areas. Adequate chemical prophylaxis does not currently exist to protect against all of the potential strains of P. falciparum troops could be exposed to in the event of deployment. The high mortality and morbidity incurred as a result of malaria in Vietnam underscores the military importance of developing adequate protection and treatment of this parasite. Recent work with an experimental sporozoite vaccine may hold some promise, but it is as yet untested in an endemic population of humans. Moreover, acquired immunoincompetence may seriously mitigate the effectiveness of any vaccine and pose an obstacle to vaccine development.

P. falciparium induced immunosuppression has been observed in endemic regions where protective immunity develops slowly and may account for an observed increased incidence of viral infections and poor sero conversion to vaccines. In vitro studies performed on mononuclear cells (MNC) from human and animal models of acute malaria have suggested acquired defects in both B and T cell populations.

Progress

The following studies were performed to examine the role of cyclic adenosine monophosphate (cyclic AMP) metabolism in MNC from infected humans (P. falciparum) and rhesus monkeys (P. knowlesi and P. cynomolgyi).

Cyclic AMP can affect MNC function in several ways. Large concentrations of exogenous cyclic AMP or pharmacologic stimulation of adenylate cyclase by receptor agonists can inhibit the blastogenic response to mitogens. A sharp early rise in cyclic AMP concentration, however, has been observed in response to mitogens preceding the blastogenic response and may provide an early intracellular trigger for cell activation. Cyclic AMP can also affect surface antigen expression in a theophylline resistent sub-set of T-cells resulting in a relative increase in T-cells with receptors for the FC fragment of Ig which posses suppressor activity (Clin. Exp. Immunol. 48:231, 1982). Cyclic AMP production in normal MNC is stimulated by hormonal receptor agonists such as prostaglandins, beta agonists, and adenosine. The activated receptor is coupled to the catalytic unit by guanine nucleotide dependent stimulatory and inhibitory regulatory subunits. Forskolin is a general activator of the catalytic unit but may be susceptable to modulation by the inhibitory regulatory subunit (JBC 257:11591, 1982).

In the experiments described here we have observed a marked depression in cyclic AMP generation in MNC from malaria infected humans and monkeys which appears to be related to the degree of infection and slowly reverses following treatment. The depressed cyclic AMP response parallels the depressed mitogen response and can be partially corrected by the addition of exogenous cyclic AMP. These data suggest that a reversible defect in cyclic AMP production in malaria may in part mediate the immunosuppression seen in this infection.

Resting cyclic AMP levels in MNC from control and infected humans demonstrate a significant depression of cyclic AMP with a mean % PRBC of only 0.46%. While there is a tendency for a greater depression at high parasitemias the level is not significantly different from that observed in the low % PRBC. Cyclic AMP production was measured in cells from control and infected humans after incubation with adenosine and Forskolin alone and in combination. Both agents significantly stimulate cyclic AMP levels in control cells. The combination of adenosine and Forskolin exhibits a slight synergistic response. MNC from infected humans are not stimulated by adenosine or Forskolin alone but show a small stimulation over resting levels in combination. MNC from rhesus monkeys, like human MNC, show a significant stimulation with either adenosine or Forskolin alone and a synergistic response when added together. At low parasitemias the synergistic response of adenosine and Forskolin together is less than control cells but the difference is not significant. The response to adenosine and Forskolin alone is, however, significantly less than the control response. The blunted response is even more remarkable in cells from high % PRBC animals where the effect of adenosine and Forskolin in combination is only 10% of the control response. Adenosine stimulated cyclic AMP generation was measured in MNC from rhesus monkeys infected with P. cynomolgyi prior to infection, during active infection, and two weeks following treatment. A significant recovery of the cyclic

AMP response in the convalescent phase was observed, although far less than the pre-infection response. This data suggests either a recovery of the defect or the addition of new MNC which are not being exposed to a serum inhibitory factor.

The blastogenic response of MNC from malaria patients was determined in normal MNC incubated with pooled malaria sera to PHA, Con A, and PWM. Significant suppression of thymidine uptake is observed in MNC from infected humans. It also appears that sera from infected humans can confer a suppressed mitogen response to normal cells. Since both the cyclic AMP generation and blastogenic response was depressed in MNC from infected monkeys and humans, experiments were performed to determine if the addition of exogenous cAMP could restore any of the immune response.

This was indeed the case. MNC showed a significant restoration of the blastogenic response when MNC from infected humans were incubated with dibutyryl-cyclic AMP. Pharmacologic concentrations cyclic AMP (10^{-3} - 10^{-4}) were inhibitory to both infected and control cells but decreasing concentrations towards 10^{-7} restored the blastogenic response in malaria MNC to a significantly greater degree than control cells. The restoration of the blastogenic response was also observed with PWM, Con A and in normal MNC incubated with infected sera.

Summary and Conclusions

1. Resting cyclic AMP levels and the response to adenosine and Forskolin is depressed in both human and monkey acute malaria.

2. A cyclic AMP generation defect can be conferred to normal MNC by incubation with malaria sera.

3. The adenosine stimulated cyclic AMP response shows a partial recovery in the convalescent phase and suggests reversibility.

4. The mitogen response of MNC from infected humans is depressed but can be partially restored by the addition of exogenous cyclic AMP.

These data suggest that malaria can confer an acquired defect in cyclic AMP generation to host MNC which may result in immunosuppression of the host cell. The partial restoration of blastogenesis after treatment with exogenous cyclic AMP supports the conclusion that a defect in cyclic AMP generation exists at the level of adenylate cyclase and supports a role for cyclic AMP in maintaining a reactive MNC population. The reduced response to

Forskolin may reflect a direct effect on the adenylate cyclase catalytic subunit; however, stimulation of the guanine nucleotide inhibitory site cannot be excluded without further study.

Future Plans and Recommendations

Experiments are now planned to 1) study large numbers of malaria patients with a newly acquired fluorescent activated cell analyzer to further investigate the relationship of altered cyclic AMP production and cell surface expression; 2) evaluate experimental immunostimulates (Isoprinosine) on cAMP production and blastogenesis *in vitro* and 3) determine if pharmacological manipulation of lymphocyte cAMP production alters the immune response *in vivo* in monkeys experimentally infected with P. knowlesi malaria.

References

Wiesmann, W.P. and Webster, H.K., Defective Mononuclear Cell cAMP Metabolism in Malaria, Clin Res, 1984.

Webster, H.K. and Wiesmann, W.P., Reversible Defect in Adenosine Mediated cAMP Response in Lymphocytes in Malaria, Science, submitted for publication.

Webster, H.K. and Wiesmann, W.P., Abnormal cAMP Production Mediates Acquired Lymphocyte Dysfunction in Human Malaria - NEJM - manuscript in preparation.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA 303116	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3M161101A91C	00	118 WWQA
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) Differentiation of Mosquito Sibling Species Using Recombinant DNA Probes

12. SUBJECT AREAS
0603 Biology 0613 Microbiology

13. START DATE 83 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	0.3	28
c. TYPE	d. AMOUNT			
e. KIND OF AWARD	f. CUM/TOTAL	85	0.3	32

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME AFRIMS
b. ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS Bangkok, Thailand
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR ROSENBERG, R
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code)
21. GENERAL USE FINA	f. NAME OF ASSOCIATE INVESTIGATOR (if available) ANDRE, R
MILITARY CIVILIAN APPLICATION H	g. NAME OF ASSOCIATE INVESTIGATOR (if available) PANYIM, S

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Medical Entomology; (U) Recombinant DNA probes; (U) Mosquitoes; (U) Malaria

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The technical objective is to develop a rapid, sensitive test using labeled, cloned mosquito DNA for differentiating morphologically identical malaria vector species, particularly members of the Anopheles balabacensis complex. There is a military requirement for this research.

24. (U) The following steps will be carried out sequentially: (a) Isolation and colonization of each of the four species, (b) Preparation, analysis and cloning of DNA from each species, and (c) Selection of clones specific only for one species and testing their specificity on wild material.

25. (U) 83 10 - 84 09. Field collections to develop isoline colonies of Anopheles dirus A, B C. and D were made. The isoline of A. dirus B came from West Malaysia. Isolines were speciated by analysis of the metaphase chromosomes isolated from the F1 progeny of each wild female mosquito. Approximately 2 ug of DNA per mosquito were isolated. The isoline colony of A. dirus B was lost before enough DNA could be isolated, leaving only a colonized strain from Perlis State in West Malaysia to test for that specific DNA. Isolines of A. dirus A, C, and D provided enough DNA to prepare initial clones for specificity and sensitivity testing. Five of 500 clones from A. dirus A were analyzed and showed both sensitivity and specificity, but degradation of the clones took place. Isolations for a different isoline of A. dirus A are using a new plasmid for transformation and 5000 clones per species are being analyzed. Progress in the next year is expected to provide a highly sensitive and highly specific means for determining the taxonomic identity of the most important vector of human malaria in Thailand and other parts of Southeast Asia.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

PROJECT: 3M161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 118: Differentiation of Mosquito Sibling Species Using Recombinant DNA Probes

Principal Investigator: CPT R. Rosenberg, MSC

OBJECTIVE: To develop a rapid, sensitive test using labeled, cloned mosquito DNA for differentiating morphologically identical malaria vector species, particularly members of the Anopheles balabacensis complex.

BACKGROUND: A serious impediment to malaria control is our inability to quickly discriminate between closely related, morphologically identical Anopheles mosquito species, such as the "An. balabacenis" complex, the most important malaria vectors in SE Asia. In Thailand four species have been found, temporarily designated An. dirus forms A, B, C, and D. Are all these species equally good vectors of malaria or are some of them relatively harmless because of characteristic range, habits, or physiology? These four species can now be differentiated from each other only by chromosome banding, electrophoresis, or cross-breeding experiments; complicated, slow, expensive and impracticable methods. Consequently, the field work necessary for determining any differences in vectorial capacity has been hindered. We propose to employ recombinant DNA techniques to construct bacterial clones containing species-specific, mosquito DNA fragments and to subsequently make radioactive labeled probes to use for identifiying wild-caught specimens.

PROGRESS: DNA was successfully extracted from a new isoline of each species and the restriction products electrophoretically fingerprinted. Eight endonucleases (Alu I, Ava II, Bgl II, Cfo I, Hae III, Kpn I, Mbo II, & Taq I) differentiated each of the 4 species, confirming previous cytogenetic studies (Baimai et al 1984). Three other nucleases (Ava I, BstN I, Nae I) separated species A from the others; four (BamH I, EcoR I, Nru I, and Pst I) separated species B; Hind III separated A and D from B and C; and four (Hha I, Rsa I, Mnl I, Msp I) could not distinguish species.

The endonucleases BamH I and Hind III were used to insert fragments of species A and species B female DNA into plasmid pBR 322 which were subsequently cloned in E. coli DH I. About 13% of the first 500 clones of species A contained moderately or highly repetitive inclusions; none of these, however, when labeled with P32 hybridized only with homologous species DNA at any of the concentrations used (60pg - 60ug).

Progress has been slower than expected, partly because recombinant DNA has not previously been used to speciate metazoon animals and much empirical manipulation was necessary to find optimum technical conditions. A severe limit has been the poor rate of tranformation in pBR 322; the vector now being used is PUN 121 which contains in addition to ampicillin and tetracycline resistant regions a tetracycline repressor region. Clone formation has now quintupled.

FUTURE OBJECTIVES: We estimate 5000 clones/species will be needed to find the requisite 2-5 specific sequences. At the rate of 500/week this initial phase should be completed by mid-1985 and testing of wild-caught mosquitoes, confirmed by chromosome mapping, can begin in preparation of a practical field test.

REFERENCES:

Baimai, V., Green, C.A., Andre, R.G., Harrison, B.A. and Peyton, E.L. Cytogenetics studies of some species complexes of Anopheles in Thailand and Southeast Asia. SE Asian J. Trop. Med. Pub. Hlth. (in press).

Presentations:

1. Research on Southeast Asia Malaria Vectors (SEAMEO), 1984, Kuala Lumpur Malaysia.

2. U.S. Military Laboratories in Asia Meeting, May 1984, Thailand.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			DAOG7010	01 OCT 84	REPORT CONTROL NUMBER Z01708
01 OCT 83	H. TERMINATED	U	U	CX	A. WORK UNIT
10 NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER	
	61101A	3A161101A91C	00	124	AR
FORMER					
MISS. OBJ.	NONE				

(U) DEVELOPMENT OF SPECIFIC CELL DIRECTED ANTIBODY-TOXIN CONJUGATES

0613 MICROBIOLOGY 0603 BIOLOGY

JAN 81	SEP 84	DA	C. IN-HOUSE

EXPIRATION

F. CUM/TOT: $ 0

FISCAL YEAR	PROFESSIONAL WORKYEARS	FUNDS
1983	2.0	$ 75
1984	2.0	$ 90

368450 11

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CIVILIAN-HIGH POTENTIAL

368450 11

NAME MDRDC WALTER REED ARMY INST OF RESEARCH
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PRINCIPAL INVESTIGATOR
NAME SADOFF, J C DA
TELEPHONE 202-576-3759

ASSOCIATE INVESTIGATORS
NAME SEID, R
NAME

(U) TOXINS;(U) ANTIBODIES ;(U) MONOCLONAL ;(U) CYTOTOXICITY;

OBJECTIVE:(U) DEVELOP TECHNIQUES FOR COUPLING TOXINS TO MONOCLONAL ANTIBODIES SUCH THAT THE TOXINS ARE INTERNALIZED BY AND KILL ONLY CELLS OR PARASITES AGAINST WHICH THE ANTIBODIES ARE DIRECTED. CELL DIRECTED TOXINS HAVE POTENTIAL FOR TREATMENT OF MILITARILY IMPORTANT PARASITE AND VIRAL INFECTIONS; DISORDERS OF IMMUNE REGULATION FOLLOWING TRAUMA, EXPOSURE TO RADIATION OR CHEMICALS; AND IN TRANSPLANTATION. AN UNDERSTANDING OF TOXIN ENTRY AND BIOCHEMISTRY IS CRITICAL AND RELEVANT IN DESIGNING STRATEGIES FOR DEFENSE AGAINST BIOLOGICAL WARFARE.

APPROACH: (U) TOXINS, SUCH AS RICIN, FOLLOWING CHEMICAL MODIFICATION OR REMOVAL OF THEIR CELL BINDING (B) REGIONS WILL BE COUPLED TO MONOCLONAL ANTIBODIES AGAINST CELLS AND PARASITES. INTRACELLULAR TOXINS WITH NO B REGION, SUCH AS GELONIN, WILL ALSO BE COUPLED TO ANTIBODY. MODIFICATION AND COUPLING PROCEDURES WILL BE OPTIMIZED FOR CELL ENTRY AND DEATH.

PROGRESS: (U) 8210-8309. A TECHNIQUE FOR PURIFICATION OF THE A FRAGMENT OF RICIN USING MONOCLONAL ANTIBODIES IS IN THE DEVELOPMENT STAGE. MONOCLONAL ANTIBODIES AGAINST RICIN A CHAIN HAVE BEEN MADE. MONOCLONAL ANTI A CHAIN ANTIBODIES HAVE BEEN USED FOR AFFINITY PURIFICATION OF A CHAIN WHICH IS AN ESSENTIAL COMPONENT FOR COUPLING. THESE MONOCLONAL ANTIBODIES HAVE POTENTIAL THERAPEUTIC POTENTIAL FOR RICIN INTOXIFICATION AND DIAGNOSTIC VALUE FOR RAPID RICIN IDENTIFICATION. FOR TECHNICAL REPORT SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 OCT 82 - 30 SEP 83.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 303117
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	125 WWSD
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) Isolation and Characterization of Potential Scrub Typhus Vaccine Components

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE: 83 10
14. ESTIMATED COMPLETION DATE: Cont
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	1.0	42
85	1.0	45

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
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c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER (include area code)
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME
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b. ADDRESS
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c. NAME OF PRINCIPAL INVESTIGATOR
HEDLUND, K W
d. TELEPHONE NUMBER (include area code)
(202)-576-2146
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
RICE, R M
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION H

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Rickettsial Diseases; (U) Scrub Typhus; (U) Structure-antigenicity; (U) Vaccine

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The morbidity that occurred among U.S. troops infected with scrub typhus both in World War II and Vietnam is well recognized. At present, while we know that prior infection leads to solid homologous immunity, we do not know the nature of immunologically important components of this organism. These studies are directed at developing a vaccine to protect troops deployed throughout the Far East.

24. (U) Current technologies will be used to isolate intact scrub typhus organisms from the bulk of eucaryotic cell membranes by a variety of non-denaturing and non-destructive techniques incorporating a gentle process simultaneously coupling centrifugation and counterflow to isolate the intact rickettsiae from the host cell membranes and their degradative enzymatic components. The isolated organisms would then selectively be fractionated into its components by high pressure liquid chromatography which inturn would be evaluated for immunodominance.

25. (U) 83 10 - 84 09 Equipment and supplies necessary for this study have been ordered and most have been received and installed. Preliminary studies have begun to isolate and purify Rickettsial tsutsugamushi by elutriation. Centrifugal and flow forces are being determined which will select for rickettsiae over and above debrie contained in the sample. Key components to the chromatography system only arrived from LKB, Sweden, 8-22-84. Early ELISA studies done on antigens isolated with what equipment was available show this approach is feasible. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 125 Isolation and Characterization of
Potential Scrub Typhus Vaccine Components

Investigators:

Principals: COL Kenneth W. Hedlund, MD
MAJ Robert M. Rice, VC

Problems and Objectives:

An estimated six percent incidence of scrub typhus occurred among U.S. troops hospitalized in RVN for malaria. Barrett estimates that ten percent of all fevers of unknown origin in RVN among U.S. troops were scrub typhus related. With particular reference to maintaining fighting capacities (Baker et al.)[1] found that ninety-three percent of scrub typhus cases in U.S. troops in RVN occurred among infantry and artillery troops rather than support elements. In recent studies of endemic areas, (Brown et al.)[2] has found that scrub typhus accounts for 19.3% of the Malaysian patients hospitalized for fevers of unknown origin. Rapmund has also commenced on the remarkable resurgence in the number of reported cases of scrub typhus in Japan.

While we know that prior infection leads to solid homologous immunity, the nature of the immunologically important components of the scrub typhus organism are completely unknown. These studies are directed at isolating and characterizing rickettsial immunogens and using these components to develop a vaccine to protect troops deployed throughout the far east.

The approach to the problem of identifying which rickettsial components would be incorporated in a scrub typhus vaccine basically couples elutriation with high pressure liquid chromatography. The first method provides a way to isolate the bulk of eucaryotic membranes and degradative enzymes from the rickettsiae which have grown in tissue culture systems. High pressure chromatography then is used to fractionate the components of rickettsiae in a nondestructive fashion for examination as immunogens in a potential vaccine.

Progress:

The elutriation system was received and installed in late July, 1984.

Standard procedures for harvesting rickettsiae from tissue culture cells include scraping or trypsinizing cells from the growing surface, homogenizing and purifying and concentrating rickettsiae by differential centrifugation. The resulting harvest can vary from 10-50% concentration of host cell material which is difficult to remove even by gradient purification. Through elutriation it is feasable to separate living from dead cells and theroretically infected cells from noninfected cells. Parameters to separate viable from nonviable LS929 cells (a suspension mouse fibroblast cell line) have been determined. Preliminary studies have started to define the parameters necessary to purify rickettsial organisms by this technique. It is hoped that by elutriation viable noninfected cells and dead cells can be washed away from infected cell which when physiologically lysed will release rickettsiae while the cell membranes and nuclei will be retained resulting in rickettsia free of host cellular material.

Unfortunately, several key components of the HPLC system have only arrived from LKB, Sweden on 22 Aug 84. However, using the equipment we have had available we have preliminary evidence based on ELISA assays to suggest that this approach is technologically feasible and will, for the very first time give insight into the nature of scrub typhus immunogens.

Recommendations:

This program to identify those scrub typhus components that elicit a protective immune response is basic to the development of an effective and acceptable vaccine. We envision that this innovative application of new technologies will be profitably pursued under ILIR funding for at least two more years.

REFERENCES CITED

1. Baker, H., R. McKinney, and D. Huxoll. Rickettsial diseases indicated by serotests at the 9th Medical Laboratory Viet Nam 1967-68. Report undated. (Cited by Barret, N. in "Rickettsial Diseases and Leptospirosis." Internal Medicine in Viet Nam, Vol. II, pg. 141, 1982, Office of the Surgeon General U.S. Army.)

2. Brown, G., A. Shirai, M. Jegathesan, D. Burke, J.C. Twartz, J.P. Sanders, adn D.L. Huxoll. 1984. Febrile Illness in Malaysia - an analysis of 1629 hospitalized patients. Am. J. Trop. Med. Hyg. 33(2):311-315.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 303118
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636
3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	WWL7 126
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code) (U) Effects of Various Endorphins and their Antagonists on Regional Blood Flow in Normal Rabbits and on Rabbits in Hypovolemic Shock.

12. SUBJECT AREAS
0619 Stress physiology 0603 Biology

13. START DATE: 83 10
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-house

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	3.0	30
85	3.0	30

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
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c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER (include area code)
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS
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Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
HARMON, J W
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(202)-576-3791
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
SAMPSON, J
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
KINNEY, R

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Lab animals; (U) rats; (U) dogs; (U) pigs; (U) endorphin; (U) shock; (U) rabbit; (U) radioactive miscrspheres; (U) blood flow

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23 (U) The objective will be to follow-up the observations of Holaday and Faden of the WRAIR that endorphin antagonists such as naloxone can reverse the hypotension of hypovolemic shock in the rat. This suggest that endorphins play a role in the pathophysiology of shock. This role has been confirmed in endotoxin shock as well as in hypovolemic shock, and in dogs and pigs as well as rats. The mechanism by which they preserve blood pressure is unknown: in some situations they raise vascular resistance and in others they do not. There is military relevance in this research.

24 (U) The objective will be to utilize radioactive microspheres in a rabbit system for measuring regional blood flow. This system has been developed in the Division. It will be used to evaluate the effects of various endorphins and their antagonists on regional blood flow in normal rabbits and on rabbits in hypovolemic shock. This work will benefit from collaboration with Dr. John Holaday of the Division of Neuropsychiatry, WRAIR.

83 10 - 84 09
25 (U) A model of hypotensive shock in the awake rabbit has been developed that allows use of the microsphere technique to measure cardiac output, resistance, and distribution of blood flow. This model was difficult to develop, but is now very satisfactory. The effects of Naloxone on blood flow in hemorrhagic shock has been evaluated and an important discovery was made. That is that Naloxone reduces renal blood flow and the percentage of cardiac output to the kidney. This clinically significant finding will be presented to the Surgical Forum, American College of Surgeons 1984. The Naloxone study must be completed and a similar evaluation of the important new antishock agent TRH is planned. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 1498 83 MAR

EDITION OF MAR 68 IS OBSOLETE.

Project: 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit: 126 EFFECTS OF VARIOUS ENDORPHINS AND THEIR ANTAGONISTS ON REGIONAL BLOOD FLOW IN NORMAL RABBITS AND ON RABBITS IN HYPOVOLEMIC SHOCK

Investigators:
John W. Harmon, LTC, MC
John A.G. Sampson, CPT, MC
Richard R. Kinney, CPT, MC

Background & Objectives

Since the discovery of endorphins various studies have shown their biological importance.

B-Endorphin, a lipotropin, has been shown to be stored along with adrenocorticotropic hormone (ACTH) in storage vesicles of the anterior pituitary. In response to stress both ACTH and B-endorphin are release from these vesicles. Guillemin et al have shown that hypophysectomy abolishes this stress response.

The analgesic effect of endorphins has been well studied by Loh et al who concluded that B-endorphin is "18 to 33 times" more potent than morphine on a molar basis, and established that endorphins are endogenous opiates.

In addition to this analgesic effect, the discovery that B-endorphin infusion in rats resulted in hypotension further encouraged study of the cardiovascular effects of this compound. Holaday first revealed the importance of endorphins in shock and various authors have shown that opiate agonist blockade by the drug naloxone reverses hypotension in shock and possibly has other beneficial effects as well.

The effects of opiate blockade has been studied in both hemorrhagic and endotoxic shock. Infusion of the opiate antagonist naloxone significantly imporved arterial blood pressure and acidosis in animal shock models. The pathway of the action of naloxine is thought to be opiate blockade though the specific site of blockade is not well understood.

Opiate receptors were originally found in the central nervous system, however Pert and Synder have more recently shown opiate receptors in mammlian brain and guinea pig intestine. The finding by Vargish et al that left ventricular contractility was specifically improved by opiate antagonism suggests a possible cardiac receptor site though this is as yet uninvestigated.

In summary there is an apparent common pathway in the pathophysiology of shock through which endorphins and other endogenous opiate agonists play an important part. Opiate antagonists, specifically naloxone, have been shown to block the pathway and prevent the induction of the shock state both acutely and prophylactically through some unknown mechanism.

It is unknown to what extent the beneficial effect of endorphin blockade in shock can be attributed to improved cardiac output, increased vascular resistance or changes in distribution of the cardiac output.

The aim of this study is to more clearly define the cardiovascular effect of opiate antagonism in non-shocked and shocked animals by investigating the changes in vascular resistance, cardiac output, and organ blood flow distribution following the intravenous administration of these compounds.

Progress:

During this fiscal year a model of hypovolemic shock was developed using a conscious rabbit model. This model enabled the study of effects blood flow distribution and cardiac output. In addition to the other hemodynamic parameters, blood pressure, heart rate and systemic vascular resistance can be easily studied with this model.

The model though initially difficult to develop is now very satisfactory.

The effects of Naloxone on blood flow in hemorrhagic shock has been evaluated and an important discovery made. Naloxone was found to reduce the renal blood flow and the percentage of cardiac output to the kidneys. This clinically significant finding has been presented to the Surgical Forum, American College of Surgeons 1984.

This model enables the Division of Surgery to perform physiological studies of shock to elucidate more fully the important pharmacologic phenomenon discovered by Dr. John Holaday in the Division of Neuropsychiatry at WRAIR.

Recommendations for the future:

We are currently evaluating the hemodymanic effects of the opiate antagonist Prolectin (TRH).

The hemodynamic effects of the opiate agonists, morphine and B-endorphin have not been well evaluated and their study would greatly add to the fund of knowledge.

With new equipment we currently have at our disposal, we will be able to follow the changes in hemodynamic parameters more closely, moving toward a more clearly defined understanding of the opiate mechanism in shock. This would add to the therapeutic regimen for soldiers injured in the field.

Publications:

Sampson J, et al. Naloxone reduces renal blood flow in rabbit hemorrhagic shock. Surgical Forum 35:22-24, 1984

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 303119	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM
83 10 01	D. Change	U	U		CX	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3M161101A91C	00	127 WM9
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) Modes of Action of Antiparasitic Drugs

12. SUBJECT AREAS
0603 Biology 0615 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	1.0	46
85	1.0	50

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER (include area code)
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS
Division of Experimental Therapeutics
Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
REID, W A
d. TELEPHONE NUMBER (include area code)
(301)-427-5029
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
BAIRD, J K
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
JACKSON, J E

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Mechanism of Action; (U) Laboratory Models; (U) Biology; (U) Pharmacology; (U) Side Effects; (U) Methodology; (U) Parasitic Diseases

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Development of new procedures to assess efficacy of candidate antiparasitic drugs, investigation of modes of activity of drugs and development of techniques to examine host or parasite responses which may enhance, limit or preclude the development of the candidate drugs. There is military relevance in this research.

24. (U) Compounds which show enhanced as unusual activity against parasites against which they have not been tested previously or compounds which show unexpected results in unusual treatment regimens or in combination with other compounds will be studied in modifications of established laboratory models and technical procedures or specifically developed techniques. Modified or new procedures will be evaluated for incorporation into the overall program. Procedures will be developed for detailed study of modes of action of compounds on metabolic and structural processes of parasites and their hosts so that limits of application of compounds as drugs may be determined or opportunities for drug intervention may be recognized.

25. (U) 83 10 - 84 09. A new in vitro test system has been developed to generate quantitative data on molecular mode-of-action of hemotoxicity of antiparasitic test compounds. In preliminary tests, this in vitro technology has been successfully applied to the clinical problem of 8-aminoquinoline (i.e. primaquine) hemotoxicity. A hypothesis describing a nonoxidative hemolysis by primaquine and several primaquine metabolites was developed and tested using the in vitro system. Vigorous testing is continuing in a comprehensive 8-aminoquinoline SAR series. Verified, this hypothesis will offer heretofore unavailable information on adverse hemotoxicity of antiparasitic drugs early in drug development; thus resulting in a substantial lowering of development risk, and of cost/time expenditure. For technical report see WRAIR Progress Report 1 Oct 83 - 30 Sep 84.

DD FORM 1498, 83 MAR EDITION OF MAR 68 IS OBSOLETE.

Project: 3A161101A 91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit: 127 Modes of Action of Antiparasitic Drugs

Investigators: Baird, J.K.
Jackson, J.E.

PROBLEMS AND OBJECTIVES

Primaquine, an 8-aminoquinoline antimalarial, is currently the least toxic of approved drugs having antiparasitic activity against the exoerythrocytic (latent liver) stages of relapsing malarias, *Plasmodium vivax* and *Plasmodium ovale*. Primaquine is also frequently employed in combined drug therapy: a) of returning U.S. travellers, who because of chloroquine prophylaxis may have an undetected, or extended prepatent infection of 4-aminoquinoline-resistant *Plasmodium falciparum*; and b) as the gametocytocide chemotherapeutic component (coadministered with a 4-aminoquinoline, blood schizontocide) to prevent malarial transmission via mosquitoes. Primaquine is, however, a markedly hemotoxic drug to individuals with a genetic defect in red cell glucose metabolism, glucose-6-phosphate dehydrogenase (G6PD) deficiency, resulting in acute intravascular hemolysis. Geographic areas of endemic (and epidemic) malaria coincide with areas of highest prevalence of G6PD-deficient population. U.S. populations at risk include darker-hued Caucasian ethnic groups including Sardinians, Sephardic Jews, Greeks, Iranians, and 12% of American black males.

There are presently no *in vitro* tests available which would identify compounds most suitable for development as substitutes for the antimalarial, primaquine. One of the consequences of this technical deficiency is that a relatively large number of experimental compounds must be initially tested *in vivo*. Since there exists no reliable animal or *in vitro* model for the evaluation of test drug hemolytic toxicity, such information is obtained as late as during human clinical trials. In effect, investigation of potentially effective, nontoxic primaquine substitutes constitutes high cost and high risk chemotherapeutic drug development.

It is the objective of this work unit to identify host/parasite biochemical responses to candidate antimalarials *in vitro* which permit rational selection of test drugs of high antiparasite efficacy and minimal toxicity early in the drug development process.

PROGRESS

It is known that low G6PD activity is characteristic of primaquine-sensitive erythrocytes and appears to represent the pathological, genetically transmitted enzymatic deficiency. It had been postulated that the hemolytic consequences of primaquine administration were due to red cell oxidative injury by primaquine and/or its metabolic by-products. In normal erythrocytes under stress of oxidant drugs, the rate of NADPH regeneration can be greatly accelerated by increasing the amount of glucose metabolized by the hexose monophosphate shunt (HMS). Sufficient NADPH is therefore readily made available for reduction of oxidized glutathione and, directly and indirectly, for reduction of methemoglobin. Reduced glutathione also protects the sulfhydryl groups of hemoglobin and the sulfhydryl-containing enzymes against oxidative destruction. Primaquine-sensitive erythrocytes, on the other hand, are incapable of sufficiently rapid regeneration of NADPH because of their G6PD deficiency. Consequently, all reductive processes within the cell that depend upon NADPH are impaired. This hypothesis of oxidative damage via primaquine lead to development of two *in vitro* tests to assess oxidative RBC injury following treatment with test compounds: a) quantitative measurement of methemoglobin production, or b) quantitative measurement of HMS elevation. Unfortunately, neither *in vitro* test proved reliable or directly correlatable with the degree of drug-induced hemolysis by test compounds in clinical trials.

Research completed in the past year in this work unit has indicated that primaquine (and several of the metabolites) do not produce oxidative deterioration of red cells *in vitro*. An *in vitro* procedure capable of direct measure of oxidative injury to red cells was developed. Using this method it was determined that primaquine and several putative *in vivo* primaquine metabolites were incapable of causing an injurious oxidative attack on red cells *in vitro*. It was quantitatively inferred from our *in vitro* measurements that primaquine does not mediate oxidative cell injury *in vivo*. Thus, primaquine induced hemolysis cannot be mediated by oxidative attack mechanisms, as was formerly believed. A detailed hypothesis developed based on these experimental observations, postulates a mechanism for primaquine-induced hemolysis. At present, several experiments designed to further test this hypothesis have been conducted. (Details of experiments and hypothetical considerations are given in the publications listed.)

FUTURE OBJECTIVES

Efforts will continue to rigorously test the hypothesis for the molecular mechanism of primaquine-induced intravascular hemolysis. Verified, this hypothesis will provide a basis of factual information on possible adverse biologic effects of antiparasitic compounds and will result in development of *in vitro* test systems for reliable determination of candidate drug suitability in a time and cost effective manner early in the development process. Such an *in vitro* test will substantially lower risk to patients undergoing experimental drug therapy during drug development clinical trials. The *in vitro* test systems developed in the past year are currently being applied in a comprehensive drug structure versus activity relationship (SAR) testing program of potential primaquine substitutes. Preliminary results have shown the *in vitro* test to be in good agreement with clinically anticipated drug hemotoxicity. These results are in preparation for publication (see manuscript in preparation cited).

Publications:

Baird, J.K. and C. Lambros (1984). Effect of membrane filtration of antimalarial drug solutions on *in vitro* activity against *Plasmodium falciparum*. *Bull. W.H.O.* *62*(3):439-444.

Baird J.K., D.E. Davidson, Jr. and J.E. Decker-Jackson (1984). Oxidative activity of two dihydroxylated primaquine analogs: non-toxicity to glucose-6-phosphate dehydrogenase-deficient red blood cells *in vitro*. *Biochem. Pharmacol*.

Baird, J.K., J.E. Decker-Jackson, and D.E. Davidson, Jr. (1984) An *in vitro* micro-volume procedure for rapid measurement of erythrocytic hexose monophosphate shunt activity. *Int. J. Biochem*. (In Press)

Baird, J.K., G.J. McCormick, and C.J. Canfield (1984). Effects of nine synthetic putative metabolic derivatives of primaquine on hexose monophosphate shunt activity in intact human red blood cells *in vitro*. (In Preparation)

Baird, J.K. (1984) Methylene blue-mediated hexose monophosphate shunt activity elevation in intact human red blood cells *in vitro*: Independence from intracellular oxidative injury. *Int. J. Biochem*. (In Press)

Baird, J.K. (1984) Radiometric method for rapid estimation of severity of glucose-6-phosphate dehydrogenase deficiency. *Uses* of Radioisotopes in Parasitology (Hayungu, Ed.) U.S. Dept. of Energy and IAEC publication. (In Press)

Presentations:

Baird, J.K., D.E. Davidson, Jr. and J.E. Decker-Jackson (1983) Oxidative activity of two hydroxylated primaquine analogs: Non-toxicity to hemolytically sensitive human red blood cells *in vitro*. Blood 64 (5; suppl. 1). Proceedings of the 25th Annual Meeting of the American Society of Hematology, San Francisco, CA, Dec. 3-6. Abstract #72.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	*REPORT CONTROL SYMBOL*
	DA OG 9280	84 09 30	*DD-DR&E(AR) 636*

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	H. Term	U	U		CX	

10. NO./CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	128 WWQ9
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE *(Precede with Security Classification Code)* (U) Regulation of the Human Immune Response to Dengue Virus Infection by Auto Anti-Idiotype Antibodies

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	84 10	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	0.3	33
85	0.0	0

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME AFRIMS
b. ADDRESS *(include zip code)* Washington, D.C. 20307-5100	b. ADDRESS BANGKOK, THAILAND
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR BURKE, D S
d. TELEPHONE NUMBER *(include area code)* (202) 576-3551	d. TELEPHONE NUMBER *(include area code)*
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Virus; (U) Dengue fever; (U) Infections Diseases; (U) Anti-idotypic antibodies

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The technical objectives are: (1) to screen human hybridomas for production of anti-dengue antibodies and naturally occurring anti-idiotypic antibodies directed against idiotypic determinants on anti-dengue immunoglobulins and (2) to develop assays for detection of these anti-idiotypic antibodies in vivo in humans. There is a military requirement for research leading to a better understanding of the antibody response to acute dengue infections. These infections represent a serious hazard to troops operating in tropical areas.

24. (U) Conventional virologic and immunologic techniques are utilized as required.

25. (U) 83 10 - 84 09 Blood mononuclear leukocytes obtained from patients with acute dengue hemorrhagic fever were fused with cells of a human-derived myeloma line on thirty-five occasions. From over 10,000 wells seeded, 122 colonies emerged. Twenty-four colonies produced measurable ($\geq$ 1 ug/ml) quantities of IgM and six produced IgG. None of these antibodies measurably reacted with dengue virus proteins in either enzyme-linked immunoassays or immunofluorescent assays. Also, none bound strongly to any of a panel of mouse anti-dengue monoclonal antibodies. For technical report see Walter Reed Army Institute of Research Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

[illegible]ILABLE

Project Number: 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Title: Regulation of the Human Immune Response to Dengue Virus Infection by Auto Anti-Idiotypic Antibodies

Work Unit Number: 128

Problem: The technical objectives are:

1. To screen human hybridomas for production of naturally occurring anti-idiotypic antibodies directed against idiotypic determinants on anti-dengue immunoglobulins.

2. To produce and purify these anti-idiotypic antibodies in quantity.

3. To purify from serum the corresponding set of anti-dengue antibodies bearing these idiotypic determinants.

4. To determine if exogenously added autologous monoclonal anti-idiotype can regulate the production of idiotype bearing antibodies by in vitro cultures of peripheral blood monoclonal leukocytes from humans with acute dengue virus infection. There is a military requirement for research leading to a better understanding of the antibody response to acute dengue virus infection. These infections represent a serious hazard to troops operating in tropical areas.

Method:

Continuous lymphoblastoid UC729-6 cells were fused with peripheral blood mononuclear cells from patients with acute dengue using polyethylene glycol, and supernatant culture fluids of the resultant progeny clones were screened for antibody activity with immunoassays.

Progress:

Thirty-five fusions were done and over 10,000 wells seeded from which 122 colonies emerged. Thirty stable immunoglobulin-secreting clones were raised, 24 of which secreted IgM and 6 of which secreted IgG in concentrations ranging from 1 to 10 micrograms per milliliter. None of the immunoglobulin micrograms per milliliter. None of the immunoglobulin containing fluids had any antibody activity against dengue serotypes 1, 2, 3, or 4 when tested in sensitive antibody capture immunoassays. None of the immunoglobulin containing fluids had any anti-idiotypic activity when tested for reactivity with a panel mouse anti-dengue monoclonal antibodies.

Future Objectives:

This project is now terminated. Although many human hybridomas were successfully prepared, none had the antibody activity sought. Alternate approaches, such as the use of new cell lines as fusion partners and/or prefusion stimulation of the blood mononuclear cells, may be necessary to achieve full success.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OG 9281
2. DATE OF SUMMARY: 84 09 30
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	K. Completion	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	129 WWID
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) Protection of Gonadal Function from Cytotoxic Therapy

12. SUBJECT AREAS
0603 Biology 0616 Physiology 0615 Pharmacology

13. START DATE: 81 10
14. ESTIMATED COMPLETION DATE: 84 10
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	1.1	35
85	0.0	0

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H Jr.
d. TELEPHONE NUMBER (include area code)
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
Division of Medicine
b. ADDRESS
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
CROSBY, W H
d. TELEPHONE NUMBER (include area code)
(202)-576-3305
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
CUTTING, M A
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U)Lab Animals; (U)Mice; (U)Chemical Toxicity; (U)Radiation Damage; (U)Gonadal Protection; (U)Marrow Protection

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To determine the efficacy of hormones in protecting an organ system from exposure to toxic chemicals or radiation. If it is possible to protect reproductive organs of animals by hormonal suppression, it may also be possible to protect gonads of troops if they are exposed to chemicals of a similar nature or to radiation. The ability to protect gonads of patients undergoing chemotherapy or irradiation is also significant.

24. (U) Procedures include: small laboratory animal models exposed to radiation or chemicals; histopathologic processing of tissues; radioimmunoassay of serum hormone levels.

25. (U) 83 10 - 84 09. Experiments have been conducted using levels of radiation or cytotoxic chemical which were shown in pilot studies to cause significant gonadal damage without mortality. In male and female mice, treatment with a hormone prior to irradiation of the gonads had no protective effect at the levels of radiation and hormone used. Similarly, in experiments with cyclophosphamide, hormone treatment did not prevent gonadal damage. Although no protective effect of hormone treatments was found in the mouse reproductive system studied, other animals or organ systems may be more susceptible to hormonal suppression and protection against cytotoxic agents may be possible. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project 3A161101A91C: IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 129 Protection of Gonadal Junction from Cytotoxic Therapy

Investigators COL William H. Crosby, MC; LTC Daniel G. Wright, MC
Mary Cutting, MS

Description

The purpose of this work unit is to study the efficacy of hormones in protecting an organ system from exposure to toxic chemicals or radiation. The concepts explored in these studies are relevant to the protection of troops who may be exposed to toxic chemicals or radiation. If it is possible to use hormones to protect the reproductive organs of animals from cytotoxic damage, it may also be possible to protect the gonads of troops exposed to cytotoxic damage, it may also be possible to protect the gonads of troops exposed to cytotoxic agents. The protection of gonads of patients undergoing chemotherapy or radiation treatments is also an important problem addressed by these experiments. This work has broad relevance in that it may provide a general model to study the protection of organ systems such as the bone marrow against the actions of various toxic agents.

We have used the mouse reproductive system as a model. We conducted pilot studies to determine levels of exposure to radiation or the cytotoxic drug cyclophosphamide which cause significant damage to the gonads of male and female mice. Damage is determined by histological quantitative analyses of gonadal tissue. With the information on effective doses gained from pilot studies, experiments have been undertaken in which some animals are treated with hormones prior to irradiation or cyclophosphamide treatment. These mice are compared with those receiving no hormones to determine if the hormones protect the gonads from radiation or cyclophosphamide-induced damage.

Progress

Male and female mice injected with a hormone prior to irradiation of the gonads showed no protective effect of the hormone. Damage to the testes and ovaries was comparable to that observed in mice not receiving the hormone. Hormone treatments also failed to provide protection against gonadal damage by cyclophosphamide. Female mice treated with radiation or cyclophosphamide did not show ovarian recovery eight weeks after cytotoxic injury. The males did show recovery, based on quantitative histological analyses. This recovery was independent of the hormone treatment in these mice. While the mouse model described did not show any protection of gonadal function by hormone treatment, it could be of value to investigate this possibility in other animal model systems.

Future plans

This project terminated on 30 September 1984 because the proposed research was completed. Derivitive research, if appropriate will be transferred to other work units.

Publications

1. Chapman RM and O'Neil-Cutting MA: The effect of gonadotropin-releasing hormone analog on murine gonads exposed to cytotoxic agents (in review), 1984.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA303120	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	170 WWIW
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code) (U) Studies of Biochemical Changes in Human Red Blood Cells Infected with Plasmodia (malaria) Organisms in vitro

12. SUBJECT AREAS
0603 Biology 0616 Physiology

13. START DATE 83 10	14. ESTIMATED COMPLETION DATE Cont	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-house

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	2.0	94
c. TYPE	d. AMOUNT	85	2.0	100
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Medicine
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR WRIGHT, D G
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-3358
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) WHAUN, J M g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Antimalarial chemotherapy; (U) Red blood cells; (U) Metabolism; (U) Malaria

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To describe and understand biochemical differences between human host red blood cells and malaria parasites in order to define metabolic targets for the design of new, specific chemotherapy. Development of new antimalarial chemotherapy is of major current and historical military importance because of needs to deploy military personnel in regions of the world where malaria is endemic. Because the emergence chloroquine-resistant malaria is increasing around the world, it has become particularly important to identify alternate chemotherapy. Our approach is to define basic differences in biochemical and metabolic pathways necessary for growth or maintenance of normal function that distinguish normal red blood cells (RBC) and RBC infected with Plasmodium falciparum, the major malaria pathogen in humans. Strategies may then be developed to exploit such differences to design malaria specific anti-metabolities.

24. (U) Laboratory studies include measurement of (1) intermediates and enzyme levels of polyamine metabolism; (2) intermediates and enzymes of purine and pyrimidine salvage and interconversion pathways; (3) enzymes mediating methylation reactions; (4) effects of selected antimetabolities on these biochemical pathways. High performance liquid chromatography and suspension tissue culture of human RBC infected with P. falciparum in vitro and radiolabeling studies with metabolic precursors are principal techniques.

25. (U) 83 10 - 84 09 Studies have shown that inhibitors of ornithine decarboxylase, an enzyme which mediates polyamine biosynthesis, interfere with malaria parasite growth and development in RBC, that the heterocyclic inhibitor of protein synthesis, hemoharringtonine, has anti-malarial action in vitro with chloroquine resistant strains, that inhibitors of methylation pathways also markedly inhibit malaria parasite growth in vitro. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83 - 30 Sep 84

Project 3A161101A91C: IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 170 Studies of Biochemical Changes in Human Red Blood Cells with Plasmodia (Malaria) Organisms In Vitro

Investigators LTC June Whaun, MC; Dr. Nesbitt Brown, GS-13 (Div. of Biochemistry)

Description

The objective of this work unit is the study of the intermediary metabolism of both the normal and the malaria-infected red cell. Malaria is the major health problem in many of the developing tropical and subtropical countries of the world. Malaria control has been complicated by the emergence of chloroquine-resistant strains of Plasmodium falciparum, the major human malaria pathogen, as well as the growing resistance of the mosquito vector to insecticides and the administrative and economic problems in some of the developing countries. Because of needs or potential needs to deploy military personnel in malaria-endemic areas which include most tropical and subtropical areas of the world, malaria is a relevant problem for the U.S. military and new effective chemotherapeutic agents are urgently required for chemotherapy control.

Most of the chemotherapy approaches in the past have employed traditional methods of drug development, based on structure-function relationships. This approach, with development of analogues of chloroquine and quinine, has resulted in predictable emergence of chloroquine-analogue resistance by Plasmodium falciparum. Accordingly, a biochemical approach with emphasis on biochemical differences between host and parasite might provide an attractive alternative method for drug design. Drugs with different mechanisms of action could act as antimalarials. These drugs could inhibit at different points of metabolic pathways and yet have the same result -- the inhibition of parasite growth with the least toxic effect on the host.

Within the framework of this militarily-relevant problem of malaria, this laboratory is interested in defining the effects of parasitism in the human red cell -- in particular, the relations between purine metabolism, polyamine metabolism and the transmethylation pathways. In this way we will be able to know more about unparasitized red cells and to trace the different effects that perturbations in one pathway could have on the other pathways.

Polyamines are low molecular weight, long chain, cationic aliphatic compounds with multiple amine or imino groups. They include putrescine, spermidine, spermine and cadaverine. They are thought to be involved in a regulatory role in the growth and differentiation of cells and organs. Recent availability of antimetabolites, ornithine decarboxylase inhibitors such as D,L-a-difluoromethylornithine, as phamacological probes to study the role of polyamines in cell function, have shown *in vitro* correlation of parasite growth and polyamine levels.

Purines are necessary for synthesis of nucleic acids, folates, energy metabolism, enzyme cofactors necessary for host red cell and parasite growth and differentiation. Both the human red cell and the *plasmodium* require salvage synthesis of purines, as both are incapable of *de novo* synthesis. The red cell requires ATP synthesis whereas the parasite needs all purine nucleotides.

Transmethylation pathways have not been well-defined in the parasitized red cell *in vitro*. S-adenosylmethionine-mediated transmethylation reactions are essential for the regulation of various physiological processes such as protein synthesis, nucleic acid synthesis, folate synthesis, viral replication, cellular differentiation, chemotaxis of phagocytic white cells and polyamine metabolism. The major methylation compounds are S-adenosylhomocysteine and S-adenosylmethionine. These are maintained in precise ratios for normal growth and function. Imbalance of this ratio could result in growth inhibition and disturbance of specific cell function.

Progress

A. Experimental approaches

1. With the *in vitro Plasmodium falciparum* human red cell cultures, red cell metabolic pathways in both infected and uninfected red cells are being studied in the presence and absence of selected metabolic inhibitors.
    a) studies on purines and salvage synthesis pathways, such as ornithine decarboxylase, the first enzyme for biosynthesis of polyamines.
    b) studies on purines and salvage synthesis pathways of purines.
    c) studies on transmethylation pathways, metabolites and enzymes.

Logistical considerations necessitated informal collaborative studies with other scientists at WRAIR and elsewhere.

2. Selected new chemotherapeutic agents (inhibitors) have been employed to determine their antimalarial efficacy and to determine their mode of action. We have examined several new compounds - from pharamaceutical manufacturers both in this country and abroad - as well as compounds prepared locally.

3. Studies of polyamines have been accomplished in collaboration with N.D. Brown of the Division of Biochemistry. Polyamines have been determined on dansylated deproteinized biological extracts by an ultrasensitive automated ion pair HPLC deproteinized biological extracts by an ultrasensitiveautomated ion pair HPLC method. Ornithine decarboxylase activity has been determined by modification of the method of Janne and Williams-Ashman (*J. Biol. Chem.* *246*:1725-1732, 1971), by measuring the amount of labelled trapped radioactive carbon dioxide formed from 1-^{14}C - ornithine. Purine studies have been done by anion exchange HPLC by a modification of the method of Hartwick and Brown (*J. Chromatogr*. *112*:651-622, 1975). Novel drug adenosine analogue nucleotides were verified by Dr. R.K. Gordon after alkaline phosphatase digestion, by chromatographic analysis (HPLC) and spectral scans comparing the digests with standard compounds of known identity by means of a Hewlett Packard 1040A spectrophotometer. Studies of the transmethylation pathways, metabolites and enzymes have been done in collaboration with Dr. Peter K. Chiang. The separation of adenosylmethionine and related adenosylsulfur metabolites was achieved by HPLC on a cation-exchange column with an ammonium formate and ammonium sulfate elution gradient (Miura, *Anal*. *Biochem*, in press). S-adenosylhomocysteine hydrolase activity was determined using ^{14}C adenosine and homocysteine by the method of Kim et al. (*Archiv. Biochem. Biophys*. *226*:65-72, 1984).

B. Specific findings

1. Aspirin in donor sera is deleterious to *Plasmodium* *falciparum*-infected red cell cultures and may have contributed to observations of others of the unpredictability of malaria growth rates *in* *vitro*.

2. Plasmodia require salvage synthesis for purine metabolism and possess a unique pathway to permit conversion of hypoxanthine to inosine monophosphate (IMP) and then to adenosine monophosphate (AMP) and other adenosine nucleotides. Uninfected red cells lack the enzyme to effect this conversion of IMP to AMP. This parasite specific pathway may be inhibited by hadacidin.

3. Ornithine decarboxylase inhibition results in decreased parasite growth *in vitro*. The effect of a-difluoromethylornithine (courtesy of Merrell-Dow Research Center) is dose-dependent and irreversible.

4. Two methylthioadenosine analogues, SIBA and deaza-SIBA, putative methylation inhibitors, inhibited *Plasmodium falciparum* growth. The ED_{50} (dose at which growth was inhibited 50%) was 46 micromolar far deaza-SIBA and 43 micromolar for SIBA. Both compounds increased spermidine levels which could be reversed by added hypoxanthine.

5. Qinghaosu, recently isolated and chemically characterized by scientists in People's Republic of China from the ancient herb, qinghao or *Artemisia annua L.*, is an effective antimalarial agent with the same potency range as chloroquine in chloroquine-sensitive malaria. Qinghaosu appears to have no cross-resistance to chloroquine. The American-derived preparation has an ED_{50} of 6.8×10^{-9}M for Camp/Malay strain of *Plasmodium falciparum*. Qinghaosu perturbed polyamine metabolism. It caused a fall in putrescine. Spermidine levels rose at low doses and fell with increasing drug dose. The pattern of polyamine shifts was similar to that seen in neplanocin-A. Qinghaosu treated cultures showed no change in red cell morphology.

6. A new ornithine decarboxylase inhibitor, MDL-72,403 DA-06 (Merrell-Dow Research Labs), a methyl ester of ornithine, 3-penten-oic acid, 2,5-diamino-2-(fluoromethyl)-, methyl ester, dihydrochloride, (E)-, has been found effective *in vitro* in human malaria-infected RBC cultures with an ED_{50} of 0.6 mM, for FCR-3/Gambia strain of *Plasmodium falciparum*. Polyamine metabolism infected cultures following 48h exposure to this drug at 0.6 mM showed a 44% fall in putrescine levels, 54% fall in spermidine, compared to untreated infected cultures. Spermine levels showed no trends.

7. Neplanocin A, a novel carbocyclic analogue of adenosine with antitumor activity, is a potent agent with antimalarial activity. The ED_{50} for *Plasmodium falciparum* strain Camp/Malay and FCR-3/Gambia is approximately 3 micromolar. In infected red cells, levels of S-adenosylmethionine and S-adenosylhomocysteine rose with increasing concentrations of neplanocin A. A new metabolite, S-neplanocinylmethionine was formed. Purine studies showed two neplanocinyl nucleotides -- probably the di- and tri- phosphates in both infected and uninfected red cells. The identity of these compounds was resolved by cleavage of phosphate groups from ATP, ADP and

neplanocin A nucleotides with alkaline phosphatase and HPLC analysis of adenosine and neplanocin A by HPLC with a flow-through scanning spectrophotometer (Hewlett Packard 1040A). The digest yielded peaks identical to standard of adenosine and neoplanocin A in retention times. Spectral scans of the eluting peaks for adenosine and neplanocin A revealed maxima 259 and 261 nm respectively and minima of 227 and 231 nm respectively. Polyamine levels showed putrescine levels fell with drug exposure. S-adenosylhomocysteine hydrolase activity was potently inhibited with a K_i of 3 nanomolar compared to a K_m of 2 micromolar for adenosine.

These studies show the antimalarial action of a drug with concurrent followup of metabolites through three metabolic key pathways of growth and differentiation:

a) Purines: new nucleotide and analogues are formed;
b) Methylation pathways: new methylation intermediates are found;
c) Polyamines: growth inhibition without the usual pattern of polyamine inhibition;
d) S-adenosylhomocysteine hydrolase activity is inhibited.

Further studies are currently underway to assess other antimalarials in this same manner.

Abstracts

1. Whaun, J.M., Miura, G., Brown, N.D. and Chiang, P.K.: Neplanocin A, a carbocyclic analogue of adenosine, inhibits methylation in P. falciparum-infected human red cell cultures. International Conference on Polyamines. Budapest, Hungary, 1984.

2. Whaun, J.M. and Brown, N.D.: A new ornithine analogue with antimalarial activity. 37th Annual Meeting of the Society of Protozoologists. Athens, Georgia, 1984.

3. Miura, G.A., Whaun, J.M., Brown, N.D., Aarbakke, J. and Chiang, P.K.: Biological effects of neplanocin A with associated changes in cellular levels of S-adenosylhomocysteine and S-adenosylmethionine. American Society of Pharmacology and Experimental Therapeutics. Indianapolis, IN, 1984.

4. Whaun, J., Brown, N., Milhous, W., Lambros, C and Klayman, D.: Qinghaosu, a potent antimalarial agent, perturbs polyamine metabolism in human malaria cultures. Polyamines:

Basic and Clinical Aspects, a Satellite Symposium of the Third International Congress on Cell Biology, Gifu, Japan, 1984.

Published articles

1. Whaun, J.M.: The effects of aspirin-containing serum in the continuous culture of P. falciparum. Journal of Protozoology 31:381-384, 1984.

2. Webster, H.K., Whaun, J.M., Walker, M.D. and Bean, T.L.: Synthesis of adenosine nuclectides from hypoxanthine by human malaria parasites (P. falciparum) in continuous erythrocyte culture: inhibition by hadacidin but not alanosine. Biochemical Pharmacology 33:1555-7, 1984.

3. Whaun, J.M. and Brown, N.D.: Ornithine decarboxylase inhibition and the malaria-infected red cell: A model for polyamine metabolism and growth. Journal of Pharmacology and Experimental Therapeutics, in press.

4. Whaun, J.M., Brown, N.D. and Chiang, P.K.; Effects of two methylthioadenosine analoques, SIBA and deaza-SIBA, on P. falciparum-infected red cells. In Malaria and the Red Cell ed. by J.W. Eaton and G.J. Brewer, pp. 143-157, Liss, New York, 1984.

5. Webster, H.K., Wiesmann, W.P., Walker, M.D., Bean, T. and Whaun, J.M.: Hypoxanthine metabolism by human malaria infectederythrocytes: focus for the design of new antimalarial drugs. In Purine Metabolism in Man - IV, Part A, ed. by C.H.M.M. Debruyn, H.A. Simmonds and M.M. Muller, pp. 219-223, Plenum Press, New York, 1984.

6. Whaun, J.M.: Chinese medicines useful as antimalarials. In A Continuing Focus on Herbal Medicine, ed. by A.R. Henderson. American Journal of Chinese Medicine, in press.

7. Whaun, J.M., Brown, N., Milhous, W., Lambros, C., Scovill, J., Lin, A. and Klayman, D.: Qinghaosu, a potent antimalarial, perturbs polyamine metabolism in human malaria cultures. In Polyamines: Basic and Clinical Aspects, ed. O. Suzuki, (Satellite Symposium of the Third International Congress on Cell Biology), VNU Science Press, Netherlands, 1984, in press.

8. Whaun, J.M., Miura, G.A., Brown, N.D., Gordon, R.K. and Chiang, P.K.: Neplanocin A: A new antimalarial agent that perturbs S-adenosylmethionine, purine and polyamine metabolism. *Journal of Pharmacology and Experimental Therapeutics*, (in review.)

PATENTS APPLIED FOR:

8/06/84: "Treatment of Malaria with Esters of Cephalotaxine"

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA303121	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	171 WWIX
b. CONTRIBUTING				
c. CONTRIBUTING	none			

11. TITLE *(Precede with Security Classification Code)* (U) Development of Prophylaxis Against Acute Cyanide Poisoning: Studies with a Canine Model

12. SUBJECT AREAS
0611 Life Support 0603 Biology 0616 Physiology

13. START DATE 83 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-house

17. CONTRACT GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	2.5	95
85	2.5	100

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)*
Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H Jr
d. TELEPHONE NUMBER *(include area code)*
(202) 576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
Division of Medicine
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WRIGHT, D G
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(202) 576-3358
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WILLIAMS, H
g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
JOHNSON, D

21. GENERAL USE
FINA
MILITARY CIVILIAN APPLICATION: H

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Cobalamins; (U) Cyanide Poisoning; (U) Cyanide Prophylaxis; (U) Lab Animals; (U) Dogs

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23.(U) To define effective methods to increase resistance against acute cyanide poisoning using an experimental canine model. In developing strategies for medical defense against rapidly lethal chemical agents such as hydrogen cyanide gas, which troops may encounter on the modern battlefield, chemical prophylaxis is an important consideration since combat conditions may prevent effective treatment once symptomatic intoxication has occurred. Agents with proven anti-cyanide effects in vivo which do not themselves produce incapacitating side effects, such as thiosulfate and cobalamins are the most promising candidates for cyanide-prophylaxis regimens, and so these agents are to be studied in particular.

24.(U) Laboratory studies involve a canine model in which female foxhounds are monitored for respiratory rate and effort, arterial blood pressure, heart rate, arterial pO_2 pCO_2 and pH, methemoglobin, cyanomethemoglobin, blood and urine cyanide anion levels. These measurements permit calculation of the ED50 for cyanide given to the animals intravenously or by inhalation in causing discrete cardiovascular/respiratory signs of early cyanide toxicity, and they allow for a determination of the protective effects of candidate prophylactic agents: hydroxycobalamin and/or sodium thiosulfate.

25.(U) 83 10 - 84 09 Antidotal effects of vitamin B-12a and combination therapy against cyanide poisoning in canine models permit a closer approximation to that of humans. These effects include measurement of cardiorespiratory and biochemical events. Quantification of total cobalamins in blood and urine via radiologic and high pressure liquid chromatographic methods permit rate studies relative to the formation of vitamin B-12 from vitamin B12a. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83-30 Sep 84.

Project 3A1611091A91C: IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 171 Development of Prophylaxis Against Acute Cyanide Poisoning: Studies with a Canine Model

Investigators Dr. Harold Williams, Ph.D. (GS-13); LTC Daniel G. Wright, MC; COL John A. Kark, MC; Deadre Johnson, MS (GS-11)

Description

Studies of the biology and biochemistry of Vitamin B_{12} have concentrated upon the use of B_{12} analogues as antidotes to acute cyanide poisoning. Although it has been recognized for some time that B_{12}a (hydroxocobalamin) may be a useful cyanide antidote, our work represents the first rigorous pharmacologic studies of this question. Our objective is to study the feasibility of using B_{12}a both as a therapeutic and a prophylactic measure against cyanide poisoning as may be encountered by military personnel during chemical warfare.

The use of cyanide gas (HCN) by a military adversary in the event of tactical warfare is considered to be a serious possibility. The feasibility of treating poisonings of troops in a combat zone is likely to be very difficult considering the rapidity with which toxicity occurs and the problems of transporting troops to an appropriate treatment facility. Therefore, the development of prophylactic measures that can be used when exposures are likely to occur is of considerable military importance.

Hydroxocobalamin (B_{12}a) avidly binds cyanide anion (CN) to form Vitamin B_{12} (CN-B_{12}) which is rapidly excreted by the kidneys if plasma levels of CN-B_{12} exceed the plasma protein binding capacity for cobalamins. It has been recognized for some time that B_{12}a might be a useful antidote against cyanide poisoning but rigorous pharmacologic studies of its use for this purpose have not been done. Our initial studies involved the use of mice and rats to define the capacity of B_{12}a administered intravenously to detoxify cyanide salt given to the animals intravenously or intrperitoneally Subsequent studies with dogs have been designed to define the pharmacokinetics of very large doses of B_{12}a administered intravenously. Dogs are being used to determine the prophylactic, antidotal effects of B_{12}a maintained at different plasma concentrations against challenge with cyanide when B_{12}a is given to the animals by itself or in combination with other agents with anti-cyanide effects (e.g. sodium thiosulfate). The emphasis of these studies is to define the feasibility of using $B_{12}a$ to increase the resistance of an individual to the toxic effects of an exposure to cyanide gas (HCN).

Progress

During this year, study of the animal model for cyanide toxicity and $B_{12}a$ protection, developed in female foxhounds, continued along lines previously outlined.

Dogs were given measured doses of CN in both the presence and absence of Cbl-OH. Various physiological responses were observed and recorded and blood and urine specimens were analyzed for total vitamin B_{12} as well as total and free CN. The efficacy of the use of Cbl-OH as an antidote was determined by giving dogs loading anc maintenance doses of this medication.

Pharmacokinetic data reported initially in report of FY 83 relative to the clearance of CN-, vitamins B_{12} and $B_{12}a$ was completed. These data include both those of biochemical and physiological bases. A modified method for measuring CN- in biological materials was developed and was presented before a national meeting of chemists and chemical engineers. The new method is more sensitive and its reaction rate is faster than the original method used.

Data generated from the study show that $B_{12}a$ does indeed bind cyanide in vivo as evidenced by lowered levels of detectable free cyanide when in the presence of $B_{12}a$ in the peripheral blood and the almost negligible amount of free cyanide in the urine. This phenomenon is much more apparent in those cases where $B_{12}a$ was given as a continuous infusion.

Findings suggest further that the reaction rate betwen cyanide and $B_{12}a$ proceeds on an order of magnitude similar to most inorganic reactions, and that the stability of the $B_{12}a$-CN molecule improves with the passage of time.

Future plans include the measurement of the rate of B_{12} formation from administered cyanide and $B_{12}a$ as an in vivo phenonmenon. These measurements will be accomplished using High Pressure Liquid Chromatography (HPLC) in conjunction with established wet chemical and radiochemical procedures. Observations in antidotal effects against cyanide by $B_{12}a$ loading, reported initially in earlier reports, will be completed. Project will terminate at end of FY85 and derivitive research transferred to other work units.

Publications

None in FY 84.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA305923	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
	A. New	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	173
b. CONTRIBUTING				
c. ~~XXXXXXXXX~~ CONTRIBUTING	None			

11. TITLE *(Precede with Security Classification Code)*
(U) Chemical Modification and X-ray Crystallography of Acetycholinesterase

12. SUBJECT AREAS
0601 Biochemistry 0703 Organic Chemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	Cont	DA	C.In-house

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		84	0.0	00
c. TYPE	d. AMOUNT			
e. KIND OF AWARD	f. CUM/TOTAL	85	0.5	10

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)* Washington, D.C. 20307 - 5100	b. ADDRESS Division of Biochemistry Washington, D.C. 20307 - 5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F. H. Jr	c. NAME OF PRINCIPAL INVESTIGATOR Doctor, B P
d. TELEPHONE NUMBER *(include area code)* (202) 576-3551	d. TELEPHONE NUMBER *(include area code)* (202) 576-3001
21. GENERAL USE FINA	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) AchE; (U) X-ray crystallography; (U) Active Site (U) Amino Acid

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The objective of this project is to map the active site topography of the enzyme acetylcholinesterase. By obtaining the knowledge from such investigations one can understand the conformational alterations that may occur when irreversible inhibitor is covalently linked to it and also one would be able to determine the dimension and functional group of potential compounds which can reactivate the enzyme using computer graphic methods.

24. (U) Two approaches will be tested: (a) selective modification of amino acid moiety in the region of the active site (the one that affects the catalytic activity) and (b) x-ray defraction studies on the single crystals of this enzyme. Reagents which selectively modify a specific amino acid such as trinitromethane (for tyrosine) will be used to modify the enzyme. If it affects the catalytic activity, which it does, kinetically one would determine how many tyrosine residues are modified (preliminary results imply only one molecule). The modified enzyme will be digested and tryptic peptide containing modified residue will be isolated and sequenced. Similar experiments with other reagents which affect other amino acid residues will be carried out. The results obtained from these experiments will allow one to put together region of molecule which contains the active site.

The three dimensional parameters of this region will be elucidated by means of x-ray defraction studies on single crystals of the enzyme. The purified enzyme will be used to obtain single crystal by (a) vapor diffusion and (b) salt precipitation followed by gradual dehydration using ammonium sulfate and polyethylene glycol. The single crystals will be used to obtain x-ray defraction data and Fourier transform maps. Eventually isomorphus substitution using organophosphates will be attempted to obtain differential structure of the same molecule.

25. None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY		1. AGENCY ACCESSION DA305924	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. NEW	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	174
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	None			

11. TITLE (Precede with Security Classification Code)
(U) Genetic Exchange in Campylobacter

12. SUBJECT AREAS
0601 Biochemistry 0613 Microbiology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT	
a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	0.0	00
85	0.5	15

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, D.C. 20307 - 5100	b. ADDRESS Division of Biochemistry Washington, D.C. 20307 - 5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Gemski, P
d. TELEPHONE NUMBER (include area code) (202) 576-3551	d. TELEPHONE NUMBER (include area code) (202) 576-2594
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code)
(U)genetic exchange; (U) plasmid; (U) Campylobacter; (U) DNA

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objective is to develop a genetic exchange system for Campylobacter so that genes associated with virulence factors can be manipulated. Development of such systems would prove useful for preparing vaccine candidates for immunization against diarrheal diseases caused by this newly emerging pathogen and for preparing gene probes for early detection of this pathogen. This research is of military importance.

24. (U) The approach includes disciplines of bacteriology, molecular biology and biochemistry. A search for plasmids, bacteriocins and phages will be performed in isolates of Campylobacter. Such DNA molecules will then be explored for use as a basis for developing both natural gene exchange among this group and for construction of a chimeric shuttle vector.

25. (U)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY
1. AGENCY ACCESSION: DA305925
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636
3. DATE PREV SUM'RY:
4. KIND OF SUMMARY: A. NEW
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	175
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	None			

11. TITLE (Precede with Security Classification Code)
(U) Flagellar Pocket Antigens as Potential Candidates for Vaccine Development

12. SUBJECT AREAS
0603 Biology 0613 Microbiology

13. START DATE: 84 10
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	0.0	00
85	0.5	10

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code):
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL: TOP, F H JR
d. TELEPHONE NUMBER (include area code): (202) 576-3551

20. PERFORMING ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS:
Division of Biochemistry
Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR: OLENICK, J G
d. TELEPHONE NUMBER (include area code): (202) 576-3017
f. NAME OF ASSOCIATE INVESTIGATOR (if available): GELLER, R
g. NAME OF ASSOCIATE INVESTIGATOR (if available):

21. GENERAL USE: FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Flagellar Pocket Antigens; (U) Recombinant DNA; (U) Vaccine; (U) Trypanosome

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objective is to conduct highly systematic studies that will permit a definitive evaluation of the potential of flagellar pocket antigens of Trypanosoma rhodesiense to afford immunoprotection against trypanosomiasis. Such an assessment is extremely important as a prelude to vaccine development and the protection of non-immune military personnel in strategic geographical areas of high endemicity.

24. (U) The approach embraces the disciplines of parasitology, microbiology, recombinant DNA technology and immunology. The site specificity of interaction of antiserum to Trypanosoma rhodesiense flagellar pocket antigens and the cross-reactivity with the flagellar pocket region of other variant-specific trypanosomes will be demonstrated by immunoelectron microscopy. Immunoprecipitated products of translated mRNA preparations from different variant-specific trypanosomes will be examined for the non-variable nature of components. Mouse protection experiments will be conducted using purified flagellar pocket antigens. Messenger RNA coding for protein recognized by anti-flagellar-pocket-antigen-serum will be size fractionated. Genomic and cDNA banks will be constructed in expression vectors. Purified products expressed by the clones will be tested in mouse protection experiments. Gene sequences conferring protection will be subcloned for further study and use.

25. (U) None.

DD FORM 1498 83 MAR
EDITION OF MAR 68 IS OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA305926

2. DATE OF SUMMARY: 84 10 01

REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY:

4. KIND OF SUMMARY: A. New

5. SUMMARY SCTY: U

6. WORK SECURITY: U

7. REGRADING:

8. DISB'N INSTR'N: CX

9. LEVEL OF SUM: A WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	176
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE *(Precede with Security Classification Code)*
(U) Production of T Cell Hybridomas

12. SUBJECT AREAS
0603 Biology 0613 Microbiology

13. START DATE: 84 10

14. ESTIMATED COMPLETION DATE: Cont

15. FUNDING ORGANIZATION: DA

16. PERFORMANCE METHOD: C. In-house

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	0.0	00
85	0.6	20

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)*
Washington, D C 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H, JR
d. TELEPHONE NUMBER *(include area code)*
(202) 576-3551

20. PERFORMING ORGANIZATION
a. NAME
Division of CD & I
b. ADDRESS
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Washington, D C 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
JERRELLS, T R
d. TELEPHONE NUMBER *(include area code)*
(202) 576-3658
f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
JARBOE, D L
g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Cell-mediated immunity; (U) T-cell hybridomas; (U) Antigen receptor; (U) T-cell epitopes

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Produce T-cell hybridomas with antigen receptors specific for antigens on Rickettsia tsutsugamushi to evaluate epitopes and T-cell receptor specificity responsible for stimulating cell-mediated immunity to this organism. This research is essential for the military as it will allow evaluation of subunit vaccines useful for immunization against scrub typnus and will allow selection of vaccine candidates stimulating the appropriate immune response. This research will greatly accelerate the production of a scrub typhus vaccine.

24. (U) Immune T-lymphocytes from mice convalescent from experimental scrub typhus will be fused, using existing technology, with the mouse T-lymphocyte lymphoma BW5147. Hybridomas responding to antigens on intact rickettsiae, as measured by lymphokine production (IL-2 and IFN-gamma), will be evaluated for strain specificity. Ultimately hybridomas will be produced from immune cells obtained from nonhuman primates. The individual antigens obtained by biochemical or molecular biological technology will be analyzed using these hybridomas to determine subunit antigens (epitopes) involved in the Cell-mediated immune response.

25. (U) None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA305927	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	177
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) Development of Human Monoclonal Antibodies Against Biological Threat Agents

12. SUBJECT AREAS
0603 Biology 0613 Microbiology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE Cont	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	0.0	00
85	1.5	50

19. RESPONSIBLE DOD ORGANIZATION

a. NAME
Walter Reed Army Institute of Research

b. ADDRESS (include zip code)
Washington, DC 20307-5100

c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR

d. TELEPHONE NUMBER (include area code)
(202) 576-3551

20. PERFORMING ORGANIZATION

a. NAME
Division of Communicable Disease & Immunology

b. ADDRESS
Walter Reed Army Institute of Research
Washington, DC 20307-5100

c. NAME OF PRINCIPAL INVESTIGATOR
Sadoff, J

d. TELEPHONE NUMBER (include area code)
(202) 576-3759

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

f. NAME OF ASSOCIATE INVESTIGATOR (if available)
Kaufman, B

g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Human monoclonal antibodies; (U) Biological threat agents ; (U) Hybridomas

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Produce hybridomas which secrete human monoclonal antibodies against biological threat agents. These antibodies will be produced and purified in quantities sufficient for epitope analysis and for in-vitro and in-vivo functional protective activities. Threat agents to be focussed on are Anthrax toxin, Rift Valley Fever, and Lassa Fever. Biological warfare agents are a serious threat to U.S. military personnel worldwide. Human monoclonal antibodies could be used as passive vaccines against these agents. They can also be used in development of active vaccines against the agents by defining epitopes humans respond to, probing gene libraries, and determining if purified vaccines retain the key epitopes.

24. (U) Volunteers are immunized with currently available vaccines. At appropriate times following immunization blood is drawn and peripheral blood lymphocytes are grown in culture in the presence of mitogens and/or antigens. When the cells have undergone blastogenesis they are fused to our myeloma fusion partner. Hybridomas secreting specific antibodies are cloned and expanded. Antibody is purified from supernatant cultures and tested for epitope specificity.

25. (U) None

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA305929	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY A.New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	178
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE *(Precede with Security Classification Code)*
(U) Identification of the Agent(s) Responsible for Human Non A Non B Hepatitis

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE Cont	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-house

17. CONTRACT/GRANT	
a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	0.0	00
85	2.0	50

19. RESPONSIBLE DOD ORGANIZATION

a. NAME
Walter Reed Army Institute of Research

b. ADDRESS *(include zip code)*
Washington, DC 20307-5100

c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR

d. TELEPHONE NUMBER *(include area code)*
(202) 576-3551

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

20. PERFORMING ORGANIZATION

a. NAME
Division of Communicable Diseases and Immunology

b. ADDRESS
Walter Reed Army Institute of Research
Washington, DC 20307-5100

c. NAME OF PRINCIPAL INVESTIGATOR
SJOGREN, M H

d. TELEPHONE NUMBER *(include area code)*
(202) 576-3478

f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
FEIGHNY, R J

g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
BURKE, D S

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) non A non B; (U) Hepatitis; (U) Immune complexes; (U) Molecular hybridization

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The technical objective is to isolate and characterize the infectious agent(s) responsible for Non A Non B hepatitis: epidemic and post-transfusional. The Non A Non B agent(s) are responsible for a significant number of acute hepatitis cases among military personnel within the USA and abroad. Once the agent(s) are characterized, rationale stategies for disease prevention, including vaccination, will be developed.

24. (U) Samples of human sera, stools and liver will be collected from both epidemic and post-transfusion Non A Non B hepatitis patients, mostly from Asia and USA. They will be screened by immune electron microscopy and by solid-phase radioimmunoassay to detect cross reactivity between sera and samples. Samples which show a potential source of Non A Non B agent(s) will be assayed for infectivity in primates. The nucleic acids will be extracted from these samples. Radiolabeled DNA probes will be prepared from both nick-translated DNA and from randomly primed RNA templates, and used for molecular hybridization studies of sera and tissue samples by Southern and Northern blotting techniques.

25. (U) None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA305931	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
	A. New	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	179
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE *(Precede with Security Classification Code)*
(U) Identification and Characterization of Meningococcal Common Antigens

12. SUBJECT AREAS
0603 Biology 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	Cont	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	0.0	00
85	1.0	25

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of Communicable Disease & Immunology
b. ADDRESS *(include zip code)* Washington, DC 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Zollinger, W D
d. TELEPHONE NUMBER *(include area code)* (202) 576-3551	d. TELEPHONE NUMBER *(include area code)* (202) 576-3651
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Seid, R g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) meningococcal; (U) monoclonal antibodies; (U) common antigens; (U) vaccine

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Identify common and cross reactive meningococcal surface antigens which are shared by all group B strains. Purify these antigens and evaluate their potential for use as a natural or synthetic vaccine against group B meningococcal disease. The ultimate objective is the development of a vaccine that is effective against all group B meningococci regardless of serotype. Military recruits during basic training are at increased risk of acquiring meningococcal disease, and the vaccine currently in use is not effective against group B strains.

24. (U) Hybridoma technology will be used to produce monoclonal antibodies which bind to all group B strains. The antigens to which these antibodies bind will be identified, purified, and characterized. The capacity of these antigens to induce bactericidal antibodies in animals and to react with bactericidal antibodies in huma sera will be determined.

25. (U) None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY		1. AGENCY ACCESSION DA305934	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	180
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)
(U) Measurement and mechanisms of immunosuppression by military stressors

12. SUBJECT AREAS
Pharmacology Stress Physiology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	0.0	00
c. TYPE	d. AMOUNT	85	1.5	75
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Neuropsychiatry
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Bernton, E
d. TELEPHONE NUMBER (include area code) (202) 576-3551	d. TELEPHONE NUMBER (include area code) (202) 576-1290
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Holaday, J g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Lab Animals; (U) Rats: (U) Immunology; (U) Stress Physiology; (U) Prolactin; (U) Endocrine

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Populations of military recruits and of mobilized soldiers are especially susceptible to a variety of infectious diseases seldom endemic in civilian populations. Acute diarrheal disease has frequently compromised operational capabilities in units recently deployed OCONUS. Basic training recruits had uniquely high attack rates for meningococcal and adenovirus disease until specific vaccine propholaxis was instituted. Data suggest that certain stressors inherent in military operations affect host susceptability to acute disease. The neuroendocrine axis and the sympathetic nervous system appear to provide mechanisms for psycho-physiologic modulation of the immune response. Prolactin is known both to be released in a graded response to stressors and to modulate immune responses. Serum prolactin levels may provide a sensitive marker for study of immuno-modulation by stressors.

24. (U) Using the primary (IgM) immune response to T-dependent and T-independent antigens, as well as viruses, as a study system, with specific IgM titers in response to immunization as an endpoint, the anterior pituitary response to antigen exposure will be further studied. Using antagonists of endogenous prolactin release, exogenous prolactin, and antagonists of endogenous opiate peptides, the effect of interventions in the neuroendocrine response to antigen exposure will then be explored. Specific stressors which elicit a neuroendocrine response will then be studied for their effect on IgM production and response to antigen . Rodents will be used for these studies.

25. (U) None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA305936		DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A WORK UNIT
	A. New	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	182
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code) (U) Development of an Assay for Measurement of Nerve Growth Factor during Development and after Neuronal Trauma and Intoxication.

12. SUBJECT AREAS
Physiology Pharmacology

13. START DATE: 84 10
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	0.0	00
85	2.5	65

19. RESPONSIBLE DOD ORGANIZATION
a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
Top, F H JR
d. TELEPHONE NUMBER (include area code)
(202) 576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
Division of Neuropsychiatry
b. ADDRESS
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
Long, J B
d. TELEPHONE NUMBER (include area code)
(202) 576-1290
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
Mobley, W C
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
Holaday, J W

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Lab Animals; (U) Rats; (U) Mice; (U) Nervous System; (U) Injury; (U) Development; (U) Enzymeimmunoassay; (U) Nerve Growth Factor

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Neuronotrophic factors influence growth, viability and biochemical and morphological characteristics of response neurons. Definition of the roles of these neuronotrophic factors in central and peripheral neuronal function will require the measurement of levels and changes in levels of these factors during development and following neuronal injury and intoxication. The technical requirements for such measurements are considerable, and to date, conventional assay techniques have proven to be inadequate. Utilizing a two site antibody recognition scheme, we shall develop an enzyme-linked radioimmunoassay for the neuronotrophic substance nerve growth factor NGF which should overcome the problems of sensitivity and specificity plagueing other assays. This assay will then be applied to examination of levels and changes in levels of NGF in physiologically relevant tissues during development, neuronal injury and recovery. This research is of military importance.

24. (U) Using NGF antibodies linked to a solid phase, NGF will be extracted from sample preparations, washed in assay buffer, and bound by a second series of NFG monoclonal antibodies from which a molecular chain consisting of a series of avidin and biotin-labeled antibodies and enzymes will be constructed. The enzyme "lactoperoxidase" will be the terminal moiety in this complex and will serve as a signal generator by catalyzing the radioiodination of the protein substrate BSA ^{125}I-BSA provides a readily measured signal, which is quantitatively proportional to the concentration of lactoperoxidase enzyme and, in turn, to the concentration of the original NGF antigen in the sample. This assay will be applied to NGF measurements in: 1) peripheral nervous tissues responsive to NGF or NGF antisera; 2) target tissue of NGF-responsive neurons; 3) brain tissues, whose associations with NGF are presently undefined. In these samples, NGF concentrations associated with different stages of neuronal injury, intoxication, and recovery in both neonatal and adult rats will be examined. Such measurements will provide important neurochemical correlates to effects of NGF or NGF antisera on neuronal function and recovery following injury.

25.(U) None.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA3059 37	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A WORK UNIT
	A.NEW	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	183
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	None			

11. TITLE *(Precede with Security Classification Code)*
(U) Pharmacologic Enhancement of Human Performance

12. SUBJECT AREAS
0510 Psychology, 0616 Physiology, 0615 Pharmacology,

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	0.0	[illegible]
85	2.5	50

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)*
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER *(include area code)*
(202) 576-3551

20. PERFORMING ORGANIZATION
a. NAME 'alter Reed Army Institute of Research
Division of Neuropsychiatry
b. ADDRESS
Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
O'DONNELL, V M
d. TELEPHONE NUMBER *(include area code)*
(301) 427-5521
f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
REDMOND, D P
g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
PLEBAN, R J

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Human Volunteer; (U) Stress; (U) Performance; (U) Sleep; (U) Drug

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The objective is to specify the effects, both positive and negative, of potential performance enhancement drugs on military performance.

24. (U) Approach is to: 1) identify variables sensitive to the effects of potential performance enhancement drugs; 2) develop laboratory and field assessment methods for the measurement of these variables; 3) assess the effects of candidate drugs on selected variables; 4) validate the variables and assessment instruments as predictors of military performance through field studies and system model simulations.

25. (U) None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA305941
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY:
4. KIND OF SUMMARY: A. New
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	184
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code) (U) Development of an EEG neonate rat model for monitoring development and plasticity of cholinergic neurons and degeneratrive CNS disease states.

12. SUBJECT AREAS
061. Pharmacology 016 Physiology

13. START DATE: 84 10
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT/GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	0.0	00
85	3.0	50

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code): Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL: Top, F H JR
d. TELEPHONE NUMBER (include area code): (202) 576-3551

20. PERFORMING ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
Division of Neuropsychiatry
b. ADDRESS: Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR: Tortella, F C
d. TELEPHONE NUMBER (include area code): (202) 576-1290
f. NAME OF ASSOCIATE INVESTIGATOR (if available): Holaday, J W
g. NAME OF ASSOCIATE INVESTIGATOR (if available): Mobley, W C

21. GENERAL USE: FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Lab Animals; (U) Rats; (U) Mice; Neuronal (U) Central Nervous System; (U) Neurotrophic Factors; (U) Electrophysiology; (U) Development

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Neurotrophic factors are soluble substances which influence the growth and viability of responsive neurons. Since most of our knowledge on the neurophysiological actions of these factors comes from experiments with peripheral nervous system neurons, our current understanding of the role of neurotrophic factors in the central nervous system (CNS) is rudimentary, at best. In order to enhance our understanding of the potential roles which these factors play in normal CNS development and during aging processes, studies directed toward a careful electrophysiological and biochemical analysis of neuronal growth and plasticity must be accomplished. We propose to investigate the electrophysiological and biochemical correlates of nerve growth factor (NGF) administration in the developing rat septo-hippocampal system, a well defined cholinergic neuronal pathway. Previous studies have demonstrated that this neuronal system responds to NGF with increases in the activity of the neurotransmitter enzyme choline acetyltransferase (ChAT). If it is possible to demonstrate that NFG accelerates synaptic function in this system, the insights gained may provide critical input for the establishment of new therapeutic approaches in degenerative CNS conditions such as Alzheimer's disease. This research is of military importance.

24. (U) The initial objective of this research will be to establish a neonatal rat model for recording spontaneous electroencephalographic (EEG) activity from cortical and subcortical sites in unrestrained pups. Rat pups will be sterotaxicallly prepared with chronic bipolar recording electrodes and intracerebroventricular cannulae. Spontaneous EEG activity will be recorded in freely moving neonates and will be sampled intermittently until full development of adult EEG rythms. After establishing baselines for normal neuronal development, it will then be possible to administer NGF and/or other cholinergic agents. The EEG data will be subjected to a sophisticated spectral analysis at various intervals during each recording episode. At the conclusion of electrophysiological monitoring, neonatal brains will be sampled for ChAT and other measures of cholinergic activity.

25. (U) None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1 AGENCY ACCESSION	2 DATE OF SUMMARY	[illegible]
	DA305942	84 10 01	DD-DR&E(AR)636

3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY	6 WORK SECURITY	7 REGRADING	8 DISB'N INSTR'N	9 LEVEL OF SUM A WORK UNIT
	A.NEW	U	U		CX	

10. NO / CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a PRIMARY	61101A	3A161101A91C	00	185
b CONTRIBUTING				
c XXXXXXXXXX	None			

11 TITLE *(Precede with Security Classification Code)*
(U) Soil Sample Survey for eggs of Baylisascaris procyonis, the Raccoon Roundworm

12 SUBJECT AREAS
0603 Biology 0606 Environmental Biology

13 START DATE	14 ESTIMATED COMPLETION DATE	15 FUNDING ORGANIZATION	16 PERFORMANCE METHOD
84 10	CONT	DA	C. IN HOUSE

17 CONTRACT / GRANT
a DATE EFFECTIVE
EXPIRATION
b CONTRACT / GRANT NUMBER
c. TYPE
d AMOUNT
e KIND OF AWARD
f CUM / TOTAL

18 RESOURCES ESTIMATE

FISCAL YEARS	a PROFESSIONAL WORK YEARS	b FUNDS *(In thousands)*
84	0.0	00
85	0.3	10

19 RESPONSIBLE DOD ORGANIZATION
a NAME
WALTER REED ARMY INSTITUTE OF RESEARCH
b ADDRESS *(include zip code)*
WASHINGTON, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER *(include area code)*
202 576-3551

20 PERFORMING ORGANIZATION
a NAME
WALTER REED ARMY INSTITUTE OF RESEARCH
b ADDRESS
DIVISION OF VETERINARY MEDICINE
WASHINGTON, DC 20307-5100
c NAME OF PRINCIPAL INVESTIGATOR
TECEC, T G
d TELEPHONE NUMBER *(include area code)*
301 427-5280
e NAME OF ASSOCIATE INVESTIGATOR *(if available)*
f NAME OF ASSOCIATE INVESTIGATOR *(if available)*

21 GENERAL USE
FINA
MILITARY / CIVILIAN APPLICATION H

22 KEYWORDS *(Precede EACH with Security Classification Code)*
(U) BAYLISASCARIS; (U) PROCYONIS; (U) RACCOON; (U) ROUNDWORM (U) Lab Animals

23 TECHNICAL OBJECTIVE 24 APPROACH 25 PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) It is my intention to determine whether or not a true hazard exists. If such a problem does exist, I intend to analyze by statistical analysis the extent of, and the comparative nature of the hazard among housing areas, recreational areas, and those areas in which raccoon dens have been located. This research is of military importance.

24. (U) I propose to collect soil samples in the vicinity of housing areas and recreational areas at Fort George G. Meade. Samples will be evaluated by approved laboratory techniques for the presence of embryonated eggs.

25. (U) None.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA305970	[illegible]	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
	A. New	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	186
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE *(Precede with Security Classification Code)*
(U)Rabbit as an Animal Model for Immune Deficiency Disease

12. SUBJECT AREAS
0603 Biology 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	Cont	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	0.0	00
85	2.0	12

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Veterinary Medicine
b. ADDRESS *(include zip code)* Walter Reed Army Institute of Research Washington, DC 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR YONUSHONIS, W P
d. TELEPHONE NUMBER *(include area code)* (202) 576-3551	d. TELEPHONE NUMBER *(include area code)* (301) 427-5186
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* ROY, M g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* LIEBENBERG, S

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Lab Animals
(U) Rabbits; (U) Oryctolagus; (U) Immunodeficiency; (U) Congenital; (U) Lymphoid hypoplasia

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23.(U)An apparent autosomal recessive trait which demonstrates itself in the homozygous state with depletion in the germinal centers of the follicular areas of gut-associated lymphoid tissue has surfaced in the rigid barrier breeding colony of rabbits at WRAIR. When heterozygous carriers are bred, the trait occurs in accordance with genetic autosomal recessive expectations. Assumed homozygous animals die of immunodeficiency resulting in acute diarrhea, dehydration and hypovolemic shock. Based upon laboratory and pedigree data, rabbits will be bred for heterozygous and homozygous immunodeficient characteristics. This population will serve as an important animal model of immune deficiency disease and will expand knowledge of the relationship of immune deficiency disease to immune complex glomerulonephritis, nephrotic syndrome and end stage renal failure. This research is of military importance.

24.(U)Tissues from normal animals in the colony and those animals which died of the trait have been examined by gross and histopathology. Pedigrees have been identified which apparently pass the trait to off-spring. Tissues will be examined for immunoglobin-bearing or immunoglobin containing cells. Results of FA microscopic studies of lymphoid and gastrointestinal tissue should define immune abnormalities. Clinical immunologic parameters will be established for suspected trait carriers. Immunoelectrophoresis will screen for serum protein abnormalities. Radial-immunodiffusion and ELISA will quantitate IgG, IgA and IgM. Direct FA staining and an FA activated cell sorter will be used to quantitate B and T lymphocytes in the peripheral blood and associated lymph tissue.

25.(U) None.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION DA305971	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX
9. LEVEL OF SUM A. WORK UNIT					

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	187
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code)

(U) Autonomic Control of Gastrointestinal Transport Functions

12. SUBJECT AREAS

0616 Physiology 0615 Pharmacology 0602 Bioengineering

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE

EXPIRATION

b. CONTRACT/GRANT NUMBER

c. TYPE

d. AMOUNT

e. KIND OF AWARD

f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	0.0	00
85	0.5	44

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of Medicine
b. ADDRESS (include zip code) Walter Reed Army Medical Center Washington, D.C. 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Tai, Y H
d. TELEPHONE NUMBER (include area code) (202) 576-3551	d. TELEPHONE NUMBER (include area code) (202) 576-3694
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Gage, T P g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Lab Animals (U) Cholinergic & Adrenergic nervous system; (U) rats (U) Rabbits; (U) Diarrhea; (U) Intestinal Transport; (U) Vesicles; (U) Current Pulse

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Evidence has shown that both chemical warfare agents and possible antidotes have effects on the autonomic control of gastrointestinal transport functions and may cause disabling gastrointestinal symptoms including nausea, vomiting, and diarrhea. Our objective is to understand the nature and mechanism of the gastrointestinal toxicity of the chemical warfare agents as well assessing the gastrointestinal effects of specific antidotes in order to develop simpler, more effective, and more efficient prophylaxis and therapeutics for exposure to chemical warfare agents.

24. (U) An initial in vivo perfusion study in animals (rats and/or rabbits) will establish the dose-physiologic response relationship of the cholinergic and adrenergic agonists. Subseguent studies with the Ussing chamber technique for transepithelial electrical parameters and solute fluxes, with the epithelial cell membrane vesicle preparations for the rate of solute uptake across the apical and basolateral cell membranes, and with the current pulse method and mathematical modelling analysis for the electrical properties of the individual cell membranes are designed to obtain information for better understanding of the nature and mechanism of the drug-induced alterations in intestinal water and solute transport.

25. (U) None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY		1. AGENCY ACCESSION DA305974	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING
8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT			

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	188
b. CONTRIBUTING				
c. CONTRIBUTING	None			

11. TITLE (Precede with Security Classification Code) (U) Pathogenesis of Hepatitis A Virus Infection in Owl Monkeys

12. SUBJECT AREAS 0603 Biology 0613 Microbiology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	0.0	00
85	1.0	100

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS Division of Pathology Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR ASHER, L V S
d. TELEPHONE NUMBER (include area code) (202) 576-3551	d. TELEPHONE NUMBER (include area code) (202) 576-2254
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) BINN, L N g. NAME OF ASSOCIATE INVESTIGATOR (if available) LeDUC, J W

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Hepatitis A Virus; (U) Pathogenesis; (U) Gastrointestinal tract; (U) Liver; (U) Humoral and Cellular Immunity; (U) Oral Immunization

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To determine the pathogenesis of hepatitis A virus infection: to identify the site(s) of viral attachment and replication, to trace the spread of virus in lymphatic and cirulatory systems and to evaluate the role of humoral and cellular immunity in liver cell damage. The results of these studies should provide valuable information concerning the pathogenesis of viral diseases of digestive system and development of more effective oral vaccine against hepatitis A virus and other enteric pathogens of military importance.

24. (U) Owl monkeys are inoculated orally with a wild strain of hepatitis A virus. At different time intervals, organs including several parts of the gastrointestinal tract, mesenteric lymph nodes, spleen, liver, pancreas, kidney and lungs are examined for virus and viral antigens. The techniques used for approaching this problem include light and electron microscopy, immunofluorescence immunohistochemical staining, in vitro culture as well as serological tests.

25. (U) None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY		1. AGENCY ACCESSION DA305975	2. DATE OF SUMMARY [illegible]	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING
8. DISB'N INSTR'N CX	9. LEVEL OF SUM A WORK UNIT			

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	189
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	None			

11. TITLE *(Precede with Security Classification Code)*
(U) Effect of Opiate Blockade on Paraplegia

12. SUBJECT AREAS
0603 Biology 0616 Physiology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	0.0	00
85	2.0	30

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)* Washington, D.C. 20307-5100	b. ADDRESS Division of Surgery Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR KINNEY, R
d. TELEPHONE NUMBER *(include area code)* (202) 576-3551	d. TELEPHONE NUMBER *(include area code)* (202) 576-3791
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* HARMON, J W g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Lab Animals (U) Rabbits
(U) Spinal Injury; (U) Opiates; (U) Paraplegia; (U) Trauma Surgery

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Irreversible paraplegia is a rare, but devastating complication of temporary aortic clamping as is necessary in a variety of surgical procedures. Faden et al (NEJM 305: 1063-7, 1981) have shown that opiate blockade improves functional results in spinal cord injury. The mechanism of this protection is not known. Our objective is to demonstrate benefit of opiate blockade for protecting animals subjected to aortic occlusion from paraplegia. This research is of military importance.

24. (U) Our approach will be to utilize the rabbit model of paraplegia after temporary aortic occlusion developed by Zivin. Functional results will be assessed as will spinal cord blood flow measured with radioactive microspheres, and serum endorphine levels determined by radioimmunoassay. This animal study could provide a rationale for a clinical trial utilizing opiate antagonists to protect surgical patients from paraplegia.

25. (U) None.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE

PROJECT 3M161102BS10

RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY		1. AGENCY ACCESSION DA 304662	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	ED	161 WWIF
b. CONTRIBUTING				
~~c. CONTRIBUTING~~	STOG 82/83 - 6.2/1			

11. TITLE (Precede with Security Classification Code) (U) Renal Function and Metabolism AFter Prolonged Exposure to Organophosphate Agents

12. SUBJECT AREAS
06 15 Pharmacology 06 16 Physiology

13. START DATE 83 05	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	3.0	363
85	5.0	337

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of Medicine Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Johnson, J P
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-2386
21. GENERAL USE FINA MILITARY CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Wiesmann, W P g. NAME OF ASSOCIATE INVESTIGATOR (if available) McNeil, J S

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Organophosphate esters; (U) Anticholinesterase; (U) Kidney Function; (U) Renal Hemodynamics; (U) Intermediary Metabolism; (U) Lab animals; (U) Dogs; (U)RAM V

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To investigate the effects of sublethal exposure to organophosphate esters, either acutely or chronically, on renal function and fluid and electrolyte homeostasis in troops.

24. (U) Clearance methods, membrane transport, tissue culture, intracellular microelectrodes, spectroscopy, enzyme kinetics.

25. (U) 83 10 - 84 09

Dose responses to Di-isopropyl-flourophosphate (DFP), a model organophosphate ester, have been performed in both dog and rat. A sublethal concentration which results in substantial inhibition of RBC and plasma acetyl cholinesterase activity has been determined. Initial studies demonstrate an effect of a single sublethal exposure on food and fluid intake GFR (transient) and Na^+ and H_2O excretion. The effects of DFP on basal and vasopressin stimulated water flow have also been studied. DFP does not affect basal H_2O permeability but markedly decreases the hydro-osmotic response to maximal dose vasopressin. Cultured epithelial cell lines have been found to be exceedingly rich in acetyl-cholinesterase activity. Since these cells exhibit transepithelial transport and hormone sensitivity in culture, they should therefore supply an ideal bioassay system for relating cellular and enzymatic effects of organophosphate agents to degree of cholinergic activation. Effort has been expended to set up a system to measure the effects of DFP on membrane potentials and cell volume in cultured cells. An in-vitro tubule preparation has been developed to study the effects of DFP on cellular metabolism and oxidative phosphorylation. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

Project: 3M161102BS10 Research on Military Disease, Injury and Health Hazards

Work Unit 161: Renal Function and Metablism After Prolonged Exposure to Organophosphate Agents

Investigators:

Principal:	LTC John P. Johnson, MC
Associates:	LTC William P. Wiesmann, MC Mr. James S. McNeil

Problems and Objectives

Organophosphate esters exert their primary toxicity through cholinesterase-inhibition and cause death acutely through nervous system and respiratory effects. Current therapeutic and prophylactic measures proposed to counter the effects of these agents center on blockade of immediate lethal actions by reversible cholinesterase inhibitors. The problem of systemic toxicity in soldiers exposed to sublethal or partially blocked agents is an area which requires exploration. Both soman and DFP (di-isopropyl-flouro-phosphate) have been shown to localize extensively in the kidney. Moreover, given the characteristics of irreversible binding to Serine residues of proteins, these agents might be expected to be wide-ranging enzyme inhibitors or cellular toxins. The effect of such agents on epithelial tissues (such as kidney) and their function is largely unknown. We proposed to evaluate the effects of such agents in acute and chronic sublethal levels on kidney function at three levels: (1) whole organ function and blood flow regulation in-vivo in an animal model, (2) epithelial tissue function in-vitro in isolated tubules, epithelial models (urinary bladder and cultured cells) and, (3) cellular level (cultured cells) enzyme activity, ionic conductivity and volume regulation. Effects will be related to the degree of cholinesterase inhibition and blockade by cholinergic antagonists at each level to determine which are mediated through cholinergic pathways and which are primary effects.

Progress

Initial dose response studies of single injections of DFP have been carried out in dog and rat and results in terms of overall functional impairment have been related to degree of inhibition of RBC and plasma cholinesterase. Initial approaches have been made for an assay of kidney cholinesterase and ATPase.

In-vitro studies have been carried out on the effects of DFP on osmotic H_2O permeability and on the hydro-osmotic response to maximal and submaximal concentrations of vasopressin. An in-vitro tubule preparation has been developed to monitor oxidative state, O_2 consumption and tissue enzyme release following DFP exposure in-vivo or in-vitro.

In-Vivo Studies

Initial studies concentrated on examination of renal function following a single dose of DFP. In dogs, DFP was given at concentrations of 0.01, 0.05, 0.10, 0.25, 0.35 and 0.50 mg/kg. Two animals at 0.35 mg/kg and one animal at 0.50 mg/kg died within two days of injection. Therefore these doses were not further studied without prior protection. At concentrations of 0.01, 0.05 and 0.10 mg/kg, DFP had no effect on plasma or RBC cholinesterase activity, no effect on creatinine clearance, urine volume, urine osmolality, fractional sodium excretion or weight. Renal functional effects were only seen at 0.25 mg/kg where animals had 47% decrease in plasma cholinesterase at one and two days post-injection and 90% inhibition at 1, 6 and 12 hours. RBC cholinesterase decreased by 70% at one hour and returned to 80% of normal by 24 hours. As RBC is a true cholinesterase, it is more likely that these changes parallel changes in renal cholinesterase activity. At this concentration of DFP, animals fed poorly with some vomiting and weight loss. A marked decrease in fractional Na+ excretion without change in urine volume and with moderate decrease in urine osmolality and creatinine clearance were found. These results argue for significant intrarenal effects beyond blood flow changes as they persisted through four days before reversing. Inhibition of cholinesterase should produce naturesis, diuresis and increase in GFR. All results are different. In the time of decrease GFR and sustained anti-naturesis, continued diuresis is inappropriate.

Results of clearance studies with rats were qualitatively similar but required larger doses of DFP. Dose responses from 0.01 to 0.50 mg/kg disclosed major effects at 0.35 to 0.50 mg/kg, doses which were lethal in dogs. Once again, renal functional changes paralleled changes in plasma cholinesterase activity and were characterized by sustained but reversible decrements in fractional Na+ excretion, urine osmolality and creatinine clearance. An inappropriate diuresis was not apparent. The results suggest tubular effects of single dose DFP and provide a workable dose range for blocked higher dosages or multiple exposure studies.

In-Vitro Studies

Initial studies were performed in toad urinary bladder to examine the effect of DFP of basal and vasopressin stimulated osmotic H_2O permeability. After control H_2O flow, bladders were incubated with or without DFP for one hour, then repeat H_2O flows were measured prior to stimulation with maximal (10^{-7}M) or submaximal (10^{-9}M) concentrations of vasopressin. DFP in concentrations ranging from 10^{-3} to 10^{-5}M had no effect on basal H_2O permeability but almost completely inhibited vasopressin stimulated hydro-osmotic H_2O flow at both maximal and submaximal concentrations. These results suggest an effect of DFP on adenylate cyclase or a distal site of vasopressin activation.

In order to examine direct tubular effects of DFP, we have developed an in-vitro tubular preparation. Rat kidneys are perfused in-situ with Ringer's and 1 gr/% Ficoll at room temperature. After paling, kidneys are flushed with collaganase, broken by stirring and centrifuged. Cortical tubules are resuspended in oxygenated Ringer's with appropriate protein concentrations and studied by spectroscopy across a wide range of the u-V spectrum with a Diode-array rapid scanning spectrophotometer. This preparation has been extensively characterized by O_2 consumption, enzyme release, vital dye uptake and relative reduction of cytochrome peaks. This preparation has been designed to examine the effects of DFP in-vivo or in-vitro on metabolic function of tubular epithelia.

Future Plans and Recommendations

It is apparent that even a single sublethal exposure to DFP results in renal functional abnormalities. Proposed studies will relate in-vivo effects to tissue cholinesterase inhibition in rat. Then the effects of repeated sublethal or higher doses with phycostigmine pretreatment will be studied in rat. The effect of vasopressin in-vivo on the inappropriate diuresis of DFP in dog will be examined. In related studies, the effect of cAMP on DFP treated tissues will be examined to determine whether AVP resistance is related to an effect on adenylate cyclase. If cAMP is effective, then adenylate cyclase activity will be studied following exposure of epithelia to DFP. Also to be studied are the effects of DFP on basal Na+ transport and H+ transport as assessed by in-vitro epithelial studies. The effects of DFP on basal metabolism will be studied in-vitro by use of the cortical tubule preparation.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA305985	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A WORK UNIT
	A. NEW	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	CP	162 WWJ5
~~b. CONTRIBUTING~~				
c. CONTRIBUTING	STOG 82/83-6.2/2			

11. TITLE (Precede with Security Classification Code)
(U) Military Stress: Sustained Operations, Sleep Deprivation, Sleep Descipline and Circadian Factors

12. SUBJECT AREAS
0510 Psychology, 0616 Physiology, 0619 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	0.0	00
85	3.5	495

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER (include area code)
202 - 576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
Division of Neuropsychiatry
b. ADDRESS
Washington DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
BELENKY, G L
d. TELEPHONE NUMBER (include area code)
301 - 427-5521
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
THORNE, D R
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
PLEBAN, R J

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Stress; (U) Biological Rhythms; (U) Chronobiology; (U) Psychophysiology; (U) Work-rest schedules; (U) Sleep Deprivation-Discipline; (U) Human Volunteers.

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Enhance basic science understanding of the temporal organization of biological functions attendant upon sustained exposure to stressors in military environments. Develop indicators of the magnitude and time-course of stressor induced behavioral and physiological disorders that are the precursors of the production of psychiatric and combat casualties.

24. (U) Execute lab studies of the effects of sleep deprivation, sleep discipline, napping and biological rhythm synchronization and desynchronization on cognitive performance, psychophysiological state, mood and soldier readiness to sustain combat. Time series analyses of behavioral, electrophysiological and biochemical measures of functioning during sustained work efforts to assess changes that precede and accompany stress responses.

25. (U) 83 10 - 84 09. Progress in 6.2 applied research on these topics during past six FYs (reported in Agency Accession DA0C6457) raised basic questions of underlying circadian osciliator mechanisms and of the particular influences of nap-taking on soldier psychological stress variables. Research in this work unit is proposed to investigate those issues on the more basic level in the laboratory.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION DA OA 6441	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636				
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AA	201 WWGA
b. CONTRIBUTING				
c. XXXXXXXX	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Viral Infections of Man

12. SUBJECT AREAS
0603 Biology 0613 Microbiology 0605 Clinical Medicine

13. START DATE: 63 08
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	3.0	601
85	3.0	643

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
FINA
MILITARY CIVILIAN APPLICATION H

22. KEYWORDS (Precede EACH with Security Classification Code)
(U)Virology; (U) Immunology; (U) Arbovirus Infections; (U) Volunteers; (U) RAMI

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23 (U) To define etiology of acute infectious diseases of special hazard to military personnel, to determine and evaluate factors influencing the occurrence, distribution, severity and medical result of human virus infections, and to develop means for reducing disability due to virus diseases.

24 (U) Contemporary virological and immunological methods are applied to disease problems occurring in troops or in susceptible civilian populations in strategically important areas. New conceptual approaches and methods are developed as needed.

25 (U) 83 10 - 84 09. Monoclonal antibodies directed against a glycoprotein (NV2) found only on immature or intracellular virus, was found to be a potent infection-enhancing antibody. Such antibodies also found to be produced in dengue hemorrhagic fever patients, cause an immature virus with low infection capability, to become highly infectious. Tissue factor was produced by antibody-mediated infection of freshly harvested and purified human monocytes, but not the U-937 human monocyte cell line. Vaccine candidates for dengue virus types 1 and 2, but not type 4, were enhanced in their infection of human monocytes by heterologous antibody. This laboratory parameter correlated with the high infectivity of dengue-1 and dengue-2, and the low infectivity of dengue-4 candidate vaccine for human volunteers having heterologous antibodies from a previous flavivirus infection. Etiologic, clinical, and immunologic characteristics of AIDS related complex (ARC) and AIDS were defined in collaborative studies with NIH to include the isolation of HTLV-III, the clinical spectrum of this disease, and the selective depletion of leu 3 and leu 8 circulating mononuclear cells. Family studies demonstrated heterosexual transmission of HTLV-III disease. Epidemiologically, heterosexual promiscuity has been identified as an emerging risk factor. For technical report, see Walter Reed Army Institute of Research Annual Report, 1 Oct 83 - 30 Sep 84.

Project 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 201 Viral Infections of Man

Investigators:

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Problems and Objectives

Characterization of viruses which threaten military personnel is necessary for effective disease control. Emphasis is placed on dengue viruses, but other viral diseases are studied when necessary. Basic research on dengue viruses is directed toward evaluation of the genetic lesions causing attenuation, the enhancement of virus replication by antibody, and viral reactivity with monoclonal antibodies. Human volunteer studies are conducted to evaluate the safety and immunogenicity of candidate dengue vaccines.

Progress

1. Dengue Type 1 Vaccine Trial.

A candidate dengue type 1 vaccine (45AZ5) was tested for safety and immunogenicity in two volunteers on the project ward located at USAMRIID, Fort Detrick. The candidate vaccine was derived from human serum 17672 collected on Nauru Island and provided by Dr. Leon Rosen. The virus was passaged 21 times in Fetal Rhesus Lung (FRhL2) cells; passage 14 included treatment with a mitogen, 5-azacytidine. From passage 15 on, virus was selected for vaccine production on the basis of small plaque formation and temperature sensitivity. The final product produced < 1 mm diameter plaques in LLC-MK2 cells and showed almost total growth inhibition at 39.3 C. Human testing was carried out under FDA BB-IND 1952.

The two volunteers had yellow fever neutralizing (N) antibody but were negative for N antibody to dengue-1. Both were negative for hemagglutination inhibiting (HI) antibodies to dengue-1, 2, 3, and 4 also. Each volunteer received 2.5 X 10^4 plaque forming units (PFU) of Lot 1 intramuscularly on 20 February 1984. Daily blood sampling and clinical followup revealed viremia began on Day 1 after inoculation and lasted for 12 days in one individual and 19 days in the other. Both men became febrile on day 9; elevated temperatures lasted 5 days and reached peaks of 38.9 C and 39.0 C. Coincident with the fever both men complained of headache, muscle aches, eye pain, and showed an erythematous blanching rash of the chest abdomen and extremities. Both men became leukopenic by day 10 with minimum WBC counts of 2300 and 2700 on days 12 and 13, respectively. Additionally, for the first time in any of the dengue vaccine trials, a fall in the daily platelet counts was recorded. One volunteer fell from 230,000 platelets/ml on day 0 to 123,000 on day 12; the other fell from 316,000 on day 0 to 173,000 on day 14. Although one volunteer experienced slight bleeding from the nostril on days 1, 3, 5, 6 and 11, his platelet counts ranged from 181,000 to 377,000 during that time; bleeding was attributed to dry mucus membranes in a low humidity environment. Both volunteers were considered to have unmodified dengue fever and recovered completely by day 30.

Both volunteers developed IgM antibody to Dengue-1 by ELISA on day 13. HI antibody appeared on day 15 and was broadly cross-reactive against all four dengue serotypes and yellow fever by day 30. Both men had typical secondary flavivirus immune responses.

Of particular interest were the growth characteristics of the viruses isolated from their sera. Isolates from both vaccinees were no longer temperature sensitive nor formed only small plaques. Retesting the Lot 1 vaccine virus revealed a few large plaques in only one of 4 lines of LLC-MK2 cells in which the vaccine was plaqued. It appears that the vaccine still contains a very small minority population of large plaque, virulent virus which is not expressed in all LLC-MK2 cell lines, or the vaccine virus has a very high frequency of early reversion in humans. In either cases, the vaccine preparation is unacceptable for further human testing. An alternative dengue-1 vaccine candidate is being sought.

2. Dengue Vaccine Virus Infection of Human Monocytes.

The WRAIR "S-1" dengue-2 live virus vaccine was previously shown to be 90% effective in producing neutralizing antibodies in volunteers who had previously been immunized with the antigenically related yellow fever vaccine. However, among volunteers who had no previous sensitization to a related antigen, only about half responded to the dengue-2 vaccine. It has been postulated, and supported by laboratory data, that antibodies produced by the yellow fever vaccine form immune complexes with the dengue vaccine virus which in turn infect Fc receptor bearing monocytes. In the laboratory, the DEN- 2S-1 vaccine would not infect a human monocyte cell line (U-937) or freshly drawn human monocytes in suspension culture, unless antibodies were included in the virus inoculum and the culture medium. The dengue type 1 vaccine virus would only infect these monocyte cell systems in the presence of antibodies; the type 1 vaccine is highly infectious for human volunteers previously immunized with the yellow fever vaccine (see above). On the other hand, the candidate live attenuated dengue-4 vaccine, either alone or in the presence of antibodies, did not infect human monocytes in vitro. This correlated with the low infectivity of the dengue-4 vaccine for human volunteers; only 2 out of 5 individuals exhibited serological evidence of infection. Infectivity of human monocytes will continue to be used as a biologic characterization tool for candidate vaccines.

3. Correlation Between DEN-2 Infection Enhancement Activity of Undiluted Pre- infection Sera and DHF Caused by a Secondary Infection.

The majority (80-90%) of dengue hemorrhagic fever (DHF) cases among children in Thailand have been associated with secondary infections of DEN-2 virus. However, not all who had secondary DEN-2 infection develop DHF; only approximately 3% of those who are at risk (i.e. previously infected by one or more types of dengue virus) actually develop the severe form of infection, while the rest remain asymptomatic. Epidemiological and *in vitro* experimental evidence suggest the role of antibody dependent enhancement (ADE) of DEN-2 infection in human mononuclear phagocytes. A study was undertaken to determine if ADE of DEN-2 infection is associated with occurrence of DHF and, if so, which specific characteristic of enhancing activity is the best correlate to the risk of developing DHF. We investigated DEN-2 infection enhancing activity in pre-infection sera of DHF cases in comparison to those of asymptomatic cases from both primary and secondary infections. These pre-infection sera were obtained from the main serum collection of the prospective study of dengue infection among Phibun school children, Bangkok 1980 (see AFRIMS, Annual Progress Report, 1981). Included in the study were 9 cases of secondary infection-DHF; 32 cases of sex and age matched secondary infection-asymptomatic; and 10 cases of primary infection-asymptomatic. DEN-2 infection enhancement assays were performed using the human monocytic cell line U-937 cultured in the continuous presence of test sera at various dilutions starting from 1:10. The degree of infection enhancement was measured as the percent infected U-937 cells as determined by immunofluorescent staining.

We found no DEN-2 enhancing activity among pre-infection sera of the primary infection-asymptomatic group. DEN-2 enhancement activity was found in all of the pre-infection sera of secondary infection, both asymptomatic and DHF groups. The mean enhancement titer of the asymptomatic group was higher than that of the DHF group ($P = 0.05$). The mean power of enhancement (maximum degree of enhancement found at any one dilution of each serum) of the asymptomatic group was higher than that of the DHF group ($P = 0.001$). We also compared the "peak enhancement titer" i.e. the titer at which maximum enhancement occurs, between the two groups. Likewise, the mean peak enhancement titer of the asymptomatic group was higher than that of the DHF group, ($P = 0.005$). The peak enhancement titer of pre-infection sera of the DHF group was 1:10 (the lowest dilution tested) among 7 out of 9 cases. The enhancement profile also indicated a higher degree of enhancement in dilutions < 1:10 or even undiluted. In contrast to those sera from the asymptomatic group, the maximum enhancement (peak titer) occurred mostly at dilution 1:40 or higher. Furthermore, substantial degree of virus neutralization was observed at dilutions preceding the peak enhancement.

Infection enhancement activity in an undiluted serum cannot be measured in the U-937 cell system, due to the serum toxicity to cells. We therefore developed a micro-enhancement assay using freshly isolated human elutriated monocytes grown in a small volume of undiluted serum or serum diluted 1:5 with RPMI medium with 10% FCS and antibiotics. The degree of enhancement was measured by both virus yields and percent infected cells. We detected a low level of non-specific infection enhancement in all of the pre-infection sera of the primary-asymptomatic group. No infection, or infection less than that due to non-specific enhancement, was observed in monocytes cultures in presence of 25 out of 32 undiluted sera from the secondary infection-asymptomatic group. Sera from this group exhibited infection enhancement but of a generally low magnitude. In contrast, seven out of 9 sera from the secondary infection- DHF

AVAILABLE

group exhibited enhancement of high power. These sera had DEN-2 $PRNT_{50}$ titers of 1:10 or less. Two sera had DEN-2 $PRNT_{50}$ of 600 and 640. One exhibited neutralization at the undiluted level of serum concentration while an insufficient quantity of the other was available for testing.

We concluded from the above results that 1) ADE of DEN-2 infection in human monocytes does play an important role in DHF caused by a secondary infection of DEN-2 virus and 2) high DEN-2 power of enhancement in undiluted serum is highly correlated to the development of DHF from a secondary infection.

4. Evidence That Fibronectin Non-specifically Enhances Dengue-2 Virus Infection in Human Monocytes.

Fibronectin is a high molecular weight glycoprotein (dimer with M.W. 450,000) found in mammalian plasma and associated with mammalian cells. Plasma fibronectin is known to bind with several biological substrates such as fibrin, collagen, gelatin and cell surfaces, especially those in the mononuclear phagocytic system. Plasma fibronectin has also been reported to be one of the nonspecific opsonins for phagocytosis and clearance of certain bacteria. Fibronectin has also been reported to bind with envelop glycoproteins of some viruses.

Due to its dual ability to bind with both viral glycoprotein and surfaces of monocytes, we tested fibronectin for its ability to enhance DEN-2 infection in human monocytes *in vitro*. Our results showed that purified fibronectin (courtesy of Dr. D.F. Mosher, Department of Medicine, University of Wisconsin) at the optimum concentration of 100 ug/ml continuously present in the culture medium of RPMI with 10% FCS and antibiotics enhanced DEN-2 infection of human monocytes by approximately 10X. The degree of enhancement observed was much lower than enhancement observed in the same system by flavivirus specific antibodies. Purified DEN-2 virus was shown to bind with purified fibronectin as detected by the radioimmunoassay method.

Undiluted normal human sera or plasma that have been screened and shown to be negative for flavivirus antibodies nonetheless enhanced DEN-2 infection in human monocytes. The degree of enhancement was less and could be differentiated from that observed in the antibody dependent enhancement. The enhancement effect by normal serum was not detected at serum dilutions beyond 1:20-1:50. Since normal human plasma contains approximately 250-280 ug/ml of fibronectin, we determined if removal or reduction of the levels of fibronectin would result in removal or reduction of the serum or plasma enhancing activity. Plasma samples with known content of fibronectin were passed through gelatin-4B sepharose columns (Pharmacia Fine Chemicals) for removal of fibronectin. Eluates were found to have no detectable fibronectin by the immunoprecipitation assay. These plasma were tested and found to have lost their enhancing activity or have the activity reduced by 10 fold. When we added purified fibronectin to these treated plasma at various concentrations, the enhancing activity was not restored. We viewed this observation as an indication of a requirement for certain configuration of fibronectin molecules to be effective in the opsonization process. Interaction with fibrin or collagen has been reported to confer on fibronectin molecules effectiveness in opsonization. We conclude that fibronectin may cause the non-specific infection-enhancement activity observed with flavivirus antibody negative human sera and speculate that fibronectin in human plasma may affect dengue virus infection of monocytes _in vivo_.

5. Antibody-mediated Infection Enhancement of the Intracellular or Immature Form of Dengue-2 Virus.

Dengue fever viruses can be made much more infectious for human monocytes by first reacting the virus with antibodies that do not neutralize the virus. The virus-antibody complexes then infect monocytes via their Fc receptors. Most dengue hemorrhagic fever in Thailand is caused by dengue-2 virus infections of humans who have been previously infected with another serotype of dengue virus. It appears that the heterologous dengue antibodies in these individuals react with common antigens on the infecting virus, and the resultant immune complex then infects Fc-receptor bearing cells. This concept has been supported with infection enhancement studies employing monoclonal antibodies. Antibodies directed against common antigens on all flaviviruses enhanced the infection of Fc receptor bearing cells, whereas antibodies directed against type specific neutralization epitopes did not enhance the infection of virus doses similar to, or exceeding those injected by mosquitoes. We recently discovered that monoclonal antibodies directed against a polypeptide found only on intracellular or immature forms of the virus were highly effective in enhancing the infection of human monocytes. This particular monoclonal antibody, 2H2, reacts only with the dengue complex viruses (Dengue serotypes 1 through 4), and not with other flaviviruses, using infected cell cultures as an antigen. It was observed that 2H2 reacted with the glycoprotein p20, or NV2 in radioimmune precipitation tests; there was no reaction with the structural polypeptides that are part of the mature virus. We also observed that dengue hemorrhagic fever patients have substantial anti-NV2 antibodies in their acute phase sera. These antibodies can react with the low-infectivity immature virus thereby making it highly infectious for Fc receptor bearing cells. Recombinant DNA technology might enable synthesis of vaccines that do not induce antibodies to NV2.

6. Mediators Produced by Macrophages Infected With Dengue-2 Virus.

Soluble factors produced by infected macrophages play a role in the pathophysiologic response to infection. Preliminary studies carried out in collaboration with Division of Medicine, USAMRIID, showed that tissue factor production by freshly harvested human macrophages infected with dengue-virus immune complexes was about half that of the maximum produced by macrophages treated with endotoxin. It was not possible to do these studies in the U-937 continuous human monocyte cell line; tissue factor was not detected as it was in fresh macrophages under the same experimental conditions in the same test. In collaboration with Dr. T. Allison, fresh macrophages infected with dengue immune complexes produced the same amount of platelet activating factor as did endotoxin treated control macrophages. Since the macrophage processes the infecting virus through different pathways, depending on whether the virus enters the cell through a virus receptor or as an infectious immune complex through an Fc receptor (1983 Annual Report), mediator production will be determined following each route of infection. Infection via the virus receptor requires a high multiplicity of infection in order to attain the same level of infection obtained by a low multiplicity of infection when the virus inoculum is complexed with antibodies.

7. Etiologic agent, clinical features, epidemiological observations and immunological defects of AIDS related complex (ARC) and AIDS.

We studied the clinical characteristics of AIDS patients referred to WRAMC. Common clinical features included generalized lymphadenopathy, lymphopenia, anergy, oral candidasis, and other opportunistic infections. A central feature of this disease is a severe depletion of the T helper cell subpopulation of circulating mononuclear cells. In light of these clinical observations we defined a syndrome which accurately identified patients with AIDS Related Complex (ARC) prior to severe opportunistic complications. ARC is defined as 1) chronic (greater than three months duration) lymphadenopathy with nodes of at least 1 cm in diameter, involving two or more extrainguinal sites and, 2) persistent (greater than 6 weeks) absolute T helper cell depletion (less than 400 T helper cells per mm3). The recognition of these subclinical patients has significantly enhanced research efforts and directly resulted in several important observations.

a. *Etiology of ARC and AIDS.* In collaboration with Dr. Robert Gallo, National Cancer Institute (NCI) HTLV-III was successfully isolated from the peripheral blood leukocytes of 18/21 patients with ARC and 6/6 patients with AIDS. Mononuclear cells were isolated by Ficoll - Hypaque gradient fractionation and treated for 48 hours with purified phytohemagglutin (5ug/5 ml), then cultured in medium supplemented with 10% thymocyte growth factor. HTLV-III was monitored by extracellular release of reverse transcriptase activity, direct electron microscopic examination, intracellular expression of viral proteins, and transmission to fresh human lymphocytes. This frequent successful isolation of HTLV-III strongly supported its etiologic association to ARC and AIDS.

In addition to isolation of HTLV-III, a serologic test, the whole virus ELIZA, developed by NCI collaborators, documented infection in 37/37 ARC and 14/15 AIDS cases among U.S. military. All cases were confirmed by Western blots when detected antibody to HTLV-III viral structural proteins.

b. *Target cell of HTLV-III.* In collaboration with investigators at the NIH (NCI-NIAID), we demonstrated the selective depletion of T helper inducer cells (leu3+ leu8+) in patient with HTLV-III infections manifested as ARC. Using directly labeled monoclonal antibodies and multiparameter flow cytometry, we quantitated the major mononuclear leukocyte classes and their subsets in these ARC patients and normal controls. Among ARC patients the leu3+ (T helper) population of cells showed a selective decrease of the leu8+ (T helper inducer) subpopulation of cells, suggesting that in vivo antibody response should be preserved longer than in vivo cytotoxic T cell response. The leu2+ population of cells showed a relative decrease in leu8+ cells; however, this appeared to secondary to an absolute increase in leu2+ leu8- cells. Further multiparameter flow cytometry employing viral specific antibody will help explain these observations.

c. *Clinical spectrum of HTLV-III infection.* Clinical features of HTLV-III disease have been observed to include (1) asymptomatic infection, (2) ARC, (3) ARC with clinical T cell deficiency, and (4) AIDS. Clinical features common to ARC and AIDS include generalized extra- inguinal lymphadenopathy, oral candidiasis, and skin lesions. Laboratory features include circulating atypical lymphocytes, pleomorphic mononuclear cells, leukopenia, lymphopenia, T helper cell depletion and defects in delayed hypersensitivity. Evidence which support that ARC and AIDS represent part of the clinical spectrum of a common disease are as follows: 1) HTLV-III infection was documented by isolation from peripheral blood leukocytes and presence of antibody to structural proteins to HTLV-III in 14 consecutive ARC and AIDS patients; 2) ARC patients had a mean age 7 years younger than AIDS patients; 3) AIDS patients had significantly lower mean leukocyte, lymphocytes and T helper cell counts; 4) the clinical course of ARC patients is characterized by progressive depletion of circulating mononuclear cells (lymphocytes and T helper cells) and the development of oral candidiasis, defects in delayed hypersensitivity and opportunistic infections. AIDS related complex (ARC) and AIDS represent different stages of HTLV-III infection, the clinical presentation is dictated by the integrity of the T helper cell population.

d. Heterosexual transmission of HTLV-III. ARC and AIDS have been found to occur in specific subpopulations which include homosexual males, IV drug abusers, hemophiliacs, recent Haitian immigrants, and sexual contacts and newborns of the aforementioned members of high-risk groups.

The experience at WRAMC has failed to document an identified risk factor in approximately 30% of cases of ARC and AIDS. The lack of Kaposi's sarcoma, the lack of active CMV disease and the low prevalence of evidence of previous infection with syphilis are all inconsistent with promiscuous homosexual activity. A common epidemiologic factor among non-risk cases was heterosexual promiscuity, suggesting that this may be an emerging risk factor in the military population.

To assess male to female transmissibility of HTLV-III, we evaluated 7 husband/wife sets in whom the husband had been identified as an index case of ARC or AIDS. The result are summarized in Table 1.

Table 1. Heterosexual Transmission of HTLV-III Among Spouses

Family Study Number	Husband		
	Clinical Diagnosis	HTLV-III Antibody	HTLV-III Isolation
WR1	AIDS	+	+
WR2	AIDS	+	+
WR3	ARC (AIDS)	+	+
WR4	AIDS	+	+
WR5	AIDS	+	NT
WR6	ARC	+	+
WR7	ARC	+	+

Family Study Number	Wife		
	Clinical Diagnosis	HTLV-III Antibody	HTLV-III Isolation
WR1	ARC	+	+
WR2	ARC	+	NT
WR3	ARC	+	-
WR4	Normal	-	-
WR5	Normal	-	NT
WR6	Normal	+	-
WR7	Normal	-	+

5/7 wives had evidence of HTLV-III infection and 3/5 of these individuals had clinical evidence of disease. Eight children (including 5 children in whom both parents had HTLV-III infection) lacked evidence of infection. These data suggest heterosexual transmission of HTLV-III. Transmission of HTLV-III by blood and blood products is clearly of military importance, however, the occurrence of heterosexual transmission could have major impact on military populations in the future.

Recommendation

Testing of live attenuated dengue vaccine should be continued to determine the feasibility of polyvalent immunization. Increased effort should be directed toward the development of dengue vaccine using the technologies of gene cloning and molecular biology. Potential subunit vaccine must be carefully evaluated not only for their ability to stimulate neutralizing antibodies but also for their ability to stimulate enhancing antibodies. Serotype-specific oligo-peptide antigens should be prepared to improve serologic methods for epidemiologic surveillance. The possible emergence of AIDS among heterosexual populations should be closely evaluated and a rational strategies for limiting the spread of HTLV in military populations should be formulated.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OB 6513
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9. LEVEL OF SUM: A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AA	202 WWGB
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code): (U) Mechanisms of Transmission of Hepatitis Viruses
12. SUBJECT AREAS: 0603 Biology 0613 Microbiology 0605 Clinical Medicine
13. START DATE: 72 07
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House
17. CONTRACT GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	302
85	2.0	323

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code): Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL: TOP, F H JR
d. TELEPHONE NUMBER (include area code): (202) 576-3551

20. PERFORMING ORGANIZATION
a. NAME: Division of Communicable Diseases & Immunol.
Walter Reed Army Institute of Research
b. ADDRESS: Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR: BURKE, D S
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f. NAME OF ASSOCIATE INVESTIGATOR (if available):

21. GENERAL USE: FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Lab Animals; (U) RAMI; (U) Monkeys; (U) Viruses; (U) Hepatitis; (U) Antigen; (U) Immunology; (U) Volunteers

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23 (U) To define the epidemiology of hepatitis in military populations in order to establish methods for reducing disability from hepatitis. Emphasis is on developing and applying sensitive and specific assays for hepatitis viruses, antigens and antibodies and to determine factors important in resistance to disease.

24 (U) New methods for the isolation, identification and comparison of hepatitis viruses are under development. The immune response of patients with viral hepatitis is studied to define sensitive measures of infection and the critical factors relating to immunoprophylaxis. The epidemiology of hepatitis in military populations is described.

25 (U) 83 10 - 84 09. Troops assigned to Korea (attack rate 6 percent/yr), and inmates of US Disciplinary Barracks (attack rate 2 percent/yr) were found to be at high risk of infection with HBV. Excessive proportions of military health workers were found to have serum markers of prior infection with HAV, HBV, and delta agent. Low molecular weight (19 S) IgM was detected as the predominant anti-HBc isotype in 74 percent of chronic carriers of HBs. IgM anti-HBc was shown to be a sensitive marker of active HBV replication in patients with chronic HBV infections. Vaccination of six owl monkeys with a formalin inactivated HAV vaccine did not produce hepatitis nor viral shedding but did stimulate HAV neutralizing antibodies. Vaccinated monkeys resisted intravenous or oral challenge with 1 million oral infections doses of virus. Radioactive DNA probes were prepared from purified HBV cores and used to detect HBV genome in clinical serum specimens. Variation among HAV strains was detected by immunoprecipitation and by nucleic acid hybridiazation. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83 - 30 Sep 84.

Project 3M161102BS10 RESEARCH ON MILITARY DISEASE,
INJURY AND HEALTH HAZARDS

Work Unit 202 Mechanisms of Transmission of Hepatitis Viruses

Investigators:

Principal:	COL William H. Bancroft, MC LTC Donald S. Burke, MC
Associates:	MAJ Maria Sjogren, MC MAJ Robert R. Redfield, MC Dr. Leonard N. Binn, Ph.D. Dr. Walter E. Brandt, Ph.D. Mr. Hubert Cannon Mrs. Ruth H. Marchwicki SSG James Lee Sosebee SP4 Norman L. Gates SP4 Reba Harris SP4 Mark Verduin-Zgonina

Problems and Objectives

Hepatitis viruses are among the most common infectious agents responsible for serious diseases among peacetime military forces today. The potential for increased transmission and epidemic spread of some forms of hepatitis, especially hepatitis A, and perhaps some types of non-A, non-B hepatitis, exists during times of mobilization with a possible resultant loss in combat effectiveness of troops. All forms of viral hepatitis may be prevented by interruption of virus transmission or passive and/or active immunoprophylaxis, although effective immunoprophylactic measures have not been fully developed. Current objectives within this work unit include the development of improved methods of specific viral diagnosis, characterization of hepatitis viruses, the study of modes of viral transmission and evaluation of means of preventing viral hepatitis.

1. Immunogenicity of Formalin-Inactivated Hepatitis A Virus Produced in Cell Culture.

Initial studies on the production of a formalin-inactivated hepatitis A virus (HAV) vaccine were described in the previous annual report. The vaccine was prepared from BS-C-1 cells infected with the HM-175 strain of HAV and inactivated with 1:4000 formalin for 4 days at 35°C. Although each of two lots were highly immunogenic in guinea pigs, the protective capacity of the vaccine was not tested. This report summarizes further studies on the preparation of this vaccine and the protection of vaccinated owl monkeys against experimental infection.

An additional lot of vaccine was prepared and detailed studies on the inactivation of the virus were done. Using the radioimmune focus assay (RIFA), the rate of inactivation by 1:4000 formalin at 35°C was determined to be essentially exponential, with an approximate 1 million-fold loss of infectivity in 30 hours. The untreated virus preparation at 35°C was also inactivated, but at a markedly lower rate. During the 10 day period of inactivation, the infectivity of the untreated preparation fell nearly 5000-fold. Immunogenicity tests of the new lot in guinea pigs revealed that all 6 animals developed significant levels of antibody in a radioimmune assay (RIA) (HAVAB, Abbott). Thus the data obtained with this new lot was similar to that described for the original 2 lots.

To determine the protective capacity of the vaccine, 6 seronegative owl monkeys were given 3 intramuscular doses of 1.0 ml on days 0, 28, and 58. None of the vaccinated monkeys developed any overt signs of disease or excreted virus in their stools. Two vaccinated monkeys had brief elevations of ALT enzyme values and a similar incidence occurred in the unvaccinated control monkeys. Biopsy of one of the vaccinated monkeys revealed an essentially normal liver. Therefore, these enzymatic elevations were not considered to be vaccine related. After the second vaccination, 5 of the 6 monkeys developed significant antibody in the RIA and all 6 were seropositive one week after the third dose. Significant levels of these antibodies persisted for at least 2 more months when the monkeys were challenged. Neutralizing antibody tests on these post vaccination serums were also done using the radioimmune focus inhibition test. After the first dose 5 of 6 monkeys were seropositive at the 1:10 serum dilution tested and all 6 were positive after the second dose. The neutralizing antibody titers ranged from 1:640 to 1:2560 or greater after the third dose.

To determine the protective capacity of the vaccine, the vaccinated monkeys were challenged 2.5 months after the third dose. Approximately 1 million infectious doses of field HAV virus was given to 3 vaccinated monkeys by the oral route and 3 monkeys were challenged by the intravenous route. None of vaccinated monkeys developed any signs of disease, or excreted any HAV in their stools, or had elevated ALT enzyme levels indicative of viral hepatitis. In contrast, each of the 2 monkeys infected by the oral or intravenous route with the same dose shed virus in their stools for 8 to 16 days and had elevated enzyme levels for 4 to 28 days. The challenged monkeys also developed HAV antibody. The findings clearly indicated that the formalin inactivated vaccine protected owl monkeys against challenge with a large dose of field HAV virus.

2. Variation Among Isolates of HAV

In order to develop an effective vaccine against HAV, it is necessary to examine antigenic variability among strains of the virus and the effect this variation may have on the immune response. Seven different HAV isolates, which had been adopted to grow in tissue culture, were examined by both immunological methods and by nucleic acid hybridization. Virus infected cells were radiolabeled *in vivo* with ^{35}S-methionine and the polypeptides extracted. The proteins were then subjected to both two-dimensional gel analysis and immunoprecipitation. This methodology allowed the detection of significant difference in the viral protein patterns depending upon the isolate studied and the cell type the virus was grown in. These strain differences were also evident when the infected cell proteins were examined using Western blotting techniques.

In addition, the appearance of viral proteins in infected cells was studied using variable multiplicities of infection and times after infection. A growth curve was generated demonstrating the events in virus replication.

RNA fingerprinting was performed on the various isolates of HAV, using virus labelled *in vivo* with ^{32}P- orthophosphate. This method did not detect differences in HAV isolates. It is unclear whether the sensitivity of the latter method is not enough to show minor variations or if there are indeed true variations in the genome as suggested by the Western blotting techniques.

The nucleic acid of the virus was tested using recombinant DNA techniques. HAV cDNA cloned in *E. coli* (provided by Dr. John Ticehurst, NIAID) was hybridized to RNA prepared from purified virus. Depending upon the isolate of virus used, differences were detected.

3. Hepatitis Markers in Peruvian Military Health Workers and Blood Donors.

A group of 309 military health workers (HW) and a 100 volunteer blood donors (BD) from Lima, Peru were screened for serological markers of hepatitis A (HAV), hepatitis B (HBV) and hepatitis Delta infection. The HW represented different degrees of occupational exposure to blood products and patients. 224 were men and 183 were women, with a mean age of 38 years (range: 17-68). Hepatitis B surface antigen (HBsAg), antibody to HBsAg (anti-HBs), antibody to hepatitis B core antigen (anti-HBc) and antibody to hepatitis A (anti-HAV) were detected by commercial radioimmunoassays (Abbott Laboratories). Antibody to Delta hepatitis (anti-delta) was detected by radioimmunoassay.It was observed that 99% of the HW were serological positive for anti-HAV. The findings of HBsAg-positivity was 1% among the BD and 1.6% among the HW. However, when other HBV markers were considered (anti-HBs, anti-HBc) only 10% of the BD were found positive in contrast to 20% of the HW. The group of HW with the highest prevalence of HBV serological markers worked in specialties with frequent contact with blood: surgery (27%), laboratory (27%), and dentistry (26%). HW with primary patient contact showed a lower incidence of HBV serological markers: Emergency Room (10%), Intensive Care Unit (4.5%). Our findings in this South American city are strikingly similar to observations made in urban North American health workers regarding the risk of contracting hepatitis B in a hospital environment. None of the HBsAg-positive individuals were positive for anti-delta.

4. Characterization of IgM Antibody to Hepatitis B Core Antigen (IgM anti-HBc) in Chronic Hepatitis B Virus (HBV) Infections.

IgM anti-HBc is present in acute and chronic HBV infections. We described a low molecular weight form of this antibody (7S IgM, anti-HBc) in chronic type B hepatitis, distinct from the 19S IgM anti-HBc observed in acute hepatitis B. To establish the frequency of this observation and its correlation with clinical and serological parameters, we studied 90 American chronic HBsAg carriers. Characterization of IgM anti-HBc was done by rate-zonal centrifugation of sera in sucrose gradients. Results: 80 patients had detectable IgM anti-HBc and 10 did not. 73/80 demonstrated a discrete IgM anti-HBc peak: 74% had a predominant 7S and 26% a predominant 19S IgM anti-HBc. The mean duration of illness (3 yrs) and degree of abnormal liver tests (> 0.05) were the same for both 7S and 19S groups. No clinical difference was observed between patients with predominant 7S or 19S IgM anti-HBc antibodies. In 3 patients the class of IgM anti-HBc remained the same in sequential sera obtained during 3 years.

5. Detection of HBV-DNA Using a Radiolabeled DNA Probe Prepared from Hepatitis B Core Antigen (HBcAg).

In chronic hepatitis B virus (HBV) infection active viral replication has been described in patients with ongoing liver disease or in HBsAg carriers in which the disease was quiescent and it reactivated.

To better evaluate the correlation between HBV replication and other markers of infection, such as HBsAg, HBeAg and IgM anti-HBc in patients with acute and chronic HBV infection it is necessary to detect the virus DNA in sera. Presence or absence of viral DNA is a definitive indicator of viral replication.

HBV-DNA was extracted from purified HBcAg, obtained from liver of an experimentally infected chimpanzee. The DNA thus extracted was then nick-translated in vitro and used as a radiolabeled probe for molecular hybridization studies of sera obtained from patients. Commercially available HBV probes (BRL) were compared to the in-house prepared probe with good correlation when positive and negative controls were assayed; this evaluation was of importance in order to establish the usefulness of this assay as a diagnostic tool. Use of this nucleic acid hybridization is being further evaluated for in situ hybridization of HBV-DNA in liver tissue sections.

6. Assessment of HBV Risk Associated with Military Assignment to Korea.

Previous observations reported in the 1983 Annual Report documented an increased risk of HBV hospitalization among U.S. troops assigned to Korea. We assessed the relationship between HBV infection and time in Korea to determine the risk of assignment-associated infection. In 1982 the Department of Bacterial Disease performed a gonorrhea (GC) vaccine trial in Korea and provided serum for anti-HBc testing. Time in Korea was an independent risk factor for HBV infection as summarized in Table 1.

Table 1. Prevalence of Anti-HBc by Demographic Factor and Time in Korea (% positive)

Demographic Factor	Time in Korea (Months)		
	>3	4-12	>12
Race			
Caucasian	3.8	7.6	19.1
Blacks	10.5	17.6	26.7
Hispanic	4.0	15.3	16.7
Oriental	25.0	38.5	50.0
Age (years)			
<20	4.9	2.4	-
20-24	3.0	8.1	7.7
25-29	9.3	13.6	27.3
≥30	20.0	23.3	30.8
Rank			
E1-E3	3.9	7.9	20.0
E4-E6	8.9	15.6	21.0
E7-E9	28.6	33.3	36.4
Officers	11.8	13.1	15.4

Based on this point prevalence data the incidence of HBV infection was calculated to be approximately 7% per 1 year tour of duty in Korea.

The mode of acquisition of HBV infection is felt to be secondary to sexual activity with Korean females. HBV infection was positively associated with several parameters of sexual activity (history of sexually transmitted disease (STD), visit to an STD clinic during the GC study, or documented GC during the GC study). (Table 2)

Table 2. Prevalence of Anti-HBc by Time in Korea and Selected Risk Factors (% positive).

Selected Risk Factors	Total	Time in Korea		
		<3	4-12	>12
STD History				
Yes	16.2	14.6	15.8	27.5
No	7.3	4.0	7.5	16.4
STD Visit*				
Yes	11.7	5.7	13.7	37.5
No	11.1	7.5	11.4	20.8
Gonorrhea*				
Yes	13.8	8.5	13.7	44.4
No	10.9	7.1	11.5	20.0
Hepatitis History				
Yes	37.5	27.7	35.5	62.7
No	10.3	6.2	10.9	19.2

*Occurring during January - March 1983

On the basis of this data, the Armed Forces Epidemiological Board formally recommended in June 1984 that US military personnel should be vaccinated for HBV before assignment to Korea.

7. Intraprison transmission of Hepatitis B.

Point prevalence data suggesting intraprison transmission of HBV in a U.S. military prison (Ft. Leavenworth) was summarized in the 1983 Annual Progress Report. In follow-up of this data, the incidence of HBV infection was determined. Paired sera were obtained from 674 inmates in May 1982 and June 1983. The distribution of HBV events is summarized in Table 3. 564 inmates remained susceptible to HBV; however, 10 inmates acquired HBV infection during 1 year of incarceration for an annual incidence among seronegatives of 1.7%.

Table 3. Distribution of HBV Events Identified During Follow-up May 1982 - June 1983

HBV	N	%
Total HBV events	110	100.0
Acquired HBV	10	9.1
Chronic HBsAg	13	11.8
Cleared HbsAg (became immune)	31	28.2
Immune (both 1982 and 83)	56	50.9

Conclusions drawn from this prison investigation are: 1) The prison population was largely susceptible to HBV (approximately 80%), 2) the incidence of intra-prison HBV ansmission was 1.7% annually and 3) HBV is continually introduced by arriving inmates. Serosusceptibles should be protected from HBV infection by vaccination.

8. Immunogenicity and reactogenicity of low dose intradermally administered Hepatitis B vaccine.

A vaccine trial with 50 volunteers was performed to compare a 3 dose, 20ug intramuscular injection series with a 3 dose, 2ug intradermal injection series. Preliminary results were summarized in the WRAIR 1983 Annual Report. Final results at days 210 and 360 are summarized below.

Table 4	Day 210		Day 360	
	ID	IM	ID	IM
Anti-HBS P/N >2.1	24/25	25/25	23/23	22/23
Mean P/N ± 1.96 x SEM	95 ± 19	114 ± 17	84 ± 26	120 ± 22
Range of P/N	8.8-173	7.6-169	5.5-190	1.7-226

Immunogenicities of the two regimens are similar in terms of % seroconversion, mean P/N value, and range of antibody response. The duration of antibody response induced by either route remains to be determined; however, all volunteers who developed P/N responses >10 had antibody detectable at 1 year. Only 1 member of the IM group (who had a poor early antibody response) lacked antibody at 1 year. Reactogenicity by either route was limited to mild local reactions which were not objectionable. Intradermal administration of Hepatitis B vaccine is comparable to the currently recommended intramuscular regimen and if employed could offer broader and less costly HBV protection for the U.S. military.

Recommendations

The WRAIR formalin-inactivated HAV vaccine should be tested in human volunteers. Other HAV vaccines, such as live attenuated vaccines prepared at other institutions, should also be tested in humans in the near future. Cost-effective strategies for prophylaxis against HBV should be formulated and effected in high-risk military populations. Clinical specimens from patients with water-borne non-A non-B hepatitis should be investigated for identification, isolation, and characterization of viral agents.

Presentations

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2. Redfield R, Lednar W, Hayne S, Kelly P, Sjogren M, Miller R, and Bancroft W. Evidence for Intraprison Transmission of Hepatitis B. Abst. 23rd ICAAC p. 81, No. 9, 1983.

3. Redfield RR, Lemon SM, and Bancroft WH. Differential Effect of Arildone on the Replication of Hepatitis A Virus and Poliovirus in Green Monkey Kidney Cells. Abst. 23rd ICAAC p. 109, No. 156, 1983.

4. Binn LN, LeDuc JW, Marchwicki RH, Lemon SM, Trahan CJ, Staky EC, and Bancroft WH. Progress Towards an Inactivated Hepatitis A Virus Vaccine. XI International Congress for Tropical Medicine and Malaria, Calgary, Canada, Sep 16-22, 1984.

5. Ennis FA, Korane I, Binn L, Marchwicki R, and Bancroft WH. Lysis of Hepatitis A Infected Cells by Lymphocytes XI International Congress for Tropical Medicine and Malaria, Calgary, Canada, Sep 16-22, 1984.

6. Sjogren MH, and Hoofnagle J. IgM Antibody to Hepatitis B Core Antigen During the Course of Chronic Type B Hepatitis. XI International Congress for Tropical Medicine and Malaria, Calgary, Canada, Sep 16-22, 1984.

7. Redfield R. Controlled Trial of the Immunogenicity and Safety of Intradermal Administration of Inactivated Hepatitis B Vaccine. XI International Congress for Tropical Medicine and Malaria, Calgary, Canada, Sep 16-22, 1984.

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9. Burke DS. Laboratory Methods in the Diagnosis of Viral Hepatitis. Consultative Meeting of the Regional Office for South East Asia of the World Health Organization on "Research Priorities in Viral Hepatitis in Southeast Asia," 20-74 March 1984, Rangoon, Burma.

Publications

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2. Lemon, SM. Prevention of Hepatitis B Virus Infections in Military Forces. Intern. Rev. Army, Navy, Air Force Med. Serv. 56: 649-652, 1983.

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4. Srivatanakul P, Burke DS, Thanasombutt S, and Tan-ngarmtrong D. Serum Markers of Hepatitis A and B Virus Infection in Thai Patients with Primary Hepatocellular Carcinima. Thai Cancer J. 9: 113-118, 1983.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 0A 6443	84 10 01	DD-DR&E(AR) 636

3 DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D Change	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AB	203 WWG2
b. CONTRIBUTING				
c. XXXXXXXXXXX	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Bacterial Diseases of Military Importance

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
58 05	Cont	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	4.0	624
85	4.0	709

19. RESPONSIBLE DOD ORGANIZATION
a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
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21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Bacterium; (U) lab animals; (U) rats; (U) Pseudomonas aeruginosa; (U) Neisseria meningitidis; (U) RamI; (U) gonococcus

23 TECHNICAL OBJECTIVE 24 APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23(U) Studies on the etiology, ecology, epidemiology, pathogenesis, physiological, immunological and diagnostic aspects of diseases of microbial origin which are current or potential problems to military forces. Current emphasis is on control of meningococcal, gonococcal and pseudomonas infections in military forces.

24(U) Basic studies on bacterial pathogens will elucidate mechanisms of pathogenesis and result in future development of prophylactic agents.

25(U) 83 10 - 84 09. As part of the ongoing efforts in the development of immunoprophylactic measures against Pseudomonas aeruginosa and Escherichia coli, a new method was devised for making human monoclonal antibody. Murine monoclonal antibodies against Pseudomonas aeruginosa have protected against lethal challenge with homologous bacteria (3 preparations) and with heterologous challenge (1 preparation). Further analysis of core-determinants of LPS has shown the expression of the J5 antigen to be more complex than previously appreciated. In a new animal model of neutropenia, the new quinolone antibiotic ceprofloxacin was highly protective against lethal infection with Pseudomonas. (For technical report see Walter Reed Army Institute of Research Annual Report, 1 Oct 83 to 30 Sept 84).

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

Project 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 203: Bacterial Diseases of Military Importance

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Problems and Objectives

Meningococcal meningitis continues to be a problem in the military population. Although vaccines are available for many of the serogroups of meningococci, no effective vaccine is available for group B meningococcus. This organism is responsible for a number of ongoing epidemics world-wide. Gram-negative bacteremia is a relatively common complication of burn wounds, trauma and neutropenia (as may exist following chemical or radiologic exposure). Optimal antimicrobial therapy still results in close to a 30 percent mortality rate. Consequently it is desirable to develop immunotherapeutic approaches to augment conventional therapy. We have been studying the possibility of both active and passive vaccine protection as a supplement to antimicrobial therapy of gram-negative bacteria. Consequently we have been identifying virulence factors of Gram-negative bacillary pathogens and then attempting to alter the virulence by immunologic manipulation.

Gram-negative bacteremia and pneumonia present a serious threat to the survival of wounded and burned military personnel as well as personnel exposed to radiation. E. coli, P. aeruginosa and Klebsiella enterobacter comprise a majority of the strains causing serious illness. We have demonstrated previously the ability of active and passive vaccines to protect against sepsis with these organisms. We have continued to develop active vaccines against these organisms as well as hyperimmune globulin and monoclonal antibodies for use as passive prophylactic agents. Prevention of pneumoniae will involve the active and passive approach for sepsis as well as protection against colonization of the upper airway with gram negative organisms. Colonization generally precedes pneumonia in patients ill enough to require treatment in intensive care units. We are attempting to develop active and passive vaccines to protect against this colonization.

Progress

Continued work on the virulence factors in bacteremic *E. coli* has demonstrated that the LPS from the 012 serotype is unique. When a J5 monoclonal antibody is tested against all the major serotypes of *E. coli* in an immunoblot assay, it binds only to the 012 LPS. This suggests that the J5 core shares a unique epitope with the 012 LPS. Blot analysis of patient sera containing high amounts of binding antibody to J5 core determinants was done. This demonstrated that even among these patients, antibody to different J5 epitopes are made and not all epitopes are widely shared by enteric bacilli. Thus it appears that an effective J5 antisera should be against a shared epitope. K1 capsule and LPS are both virulence factors in the lethal infection of LPS-hyporesponsive mice. The requirement of the K1 capsule in this model suggests that this widely prevalent capsule may affect virulence by mechanisms other than the inhibition of phagocytosis. Monoclonal antibody to strain 12.4.4 *P. aeruginosa* delayed the onset of fatal infection in a newly developed neutropenic rat model. In collaboration with other investigators we found strong evidence that the exoenzyme S of *P. aeruginosa* may be an additional important virulence factor.

Since the most promising experimental vaccine for group B meningococcal disease is based on the outer membrane proteins, an important objective is to be able to accurately survey and monitor changes in the antigenic specificity of these proteins. Since multiple outer membrane proteins are present which exhibit antigenic variation, the production of monoclonal antibodies to these antigens was undertaken to be able to study the antigenic properties of each separate protein.

Monoclonal antibodies against the major outer membrane proteins of meningococcal group B serotypes 1, 6, and 11 were produced during the past year and added to those of serotypes 2a, 2b, 2c and 15 which had been previously produced. These antibodies were used to identify and partially characterize the corresponding outer membrane proteins. The methods of sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoblotting, and immunoprecipitation were used to identify the specific protein to which each antibody bound. Strain to strain variation of antigenic specificity was observed for outer membrane proteins of classes 1, 2, 3, and 5. The immunodominant antigenic determinants of classes 1 and 5 were generally stable to boiling 2% sodium dodecyl sulfate while those of classes 2 and 3 were destroyed under the same conditions. The denaturation of these determinants was found to coincide with the dissociation of the proteins into the monomeric state. It was discovered that some of the antigenic activity of the labile determinants could be regenerated by incubation of the proteins in the zwitterionic detergent Empigen BB. Addition of Epmigen BB to the gel electrophoresis and transblot buffers allowed successful immunoblotting of these proteins.

During production of the serotype specific monoclonal antibodies hybridoma clones were also screened to identify any that were producing antibodies to cross reactive or common surface antigens. Six clones were recovered which appeared to have relatively broad crossreactivity. Three were specific for lipopolysaccharide, one for pili, one for a new, previously unrecognized, protein antigen; and the specificity of the last remains to be determined.

Work continued on the immunochemical characterization of neisserial lipooligosaccharides (LOS). The LOS of *N. meningitidis* was found to be similar to that of *Salmonella* rough mutants. A method was devised to estimate molecular weights of LPS from the meningococcus and gonococcus, by using a silver stained SDS-PAGE. This method was also used to study strain differences in LOS. The majority of strains studied had little variation in LOS composition; however, one-third of strains had 5% difference in LOS composition. IgM initiated activation of the classical complement pathway causes lysis of serum sensitive strains of N. gonorrhoeae; however, strain specific activation of the alternate pathway enhances the lysis and may account for variations in serum sensitivity of such strains. A gonococcal pilus vaccine protein was chemically conjugated to GC LPS. The chemical techniques used in conjugation did not effect the antigenic determinants of either component. The vaccine was 1000 fold less toxic than native LPS. Enhanced immunogenicity of both GC LPS and pili were shown in mice.

Previously we demonstrated that antibody against lipopolysaccharide (LPS) specific determinants could protect animals from challenge with virulent P. aeruginosa. Techniques for detoxification of the LPS by cleaving off the toxic lipid moiety from the antigenic polysaccharides were developed. These polysaccharides were then coupled to protein carriers and shown to be suitable for inducing specific antibodies in animals. Using this technique Dr. Stanley Cryz of The Swiss Serum Institute has prepared Pseudomonas LPS tetanus toxoid conjugate vaccines for human use. These vaccines are highly effective in animals and we have demonstrated that they retain the important antigenic epitopes necessary for opsonophagocytosis with human white blood cells and complement. This was done by testing the ability of opsonic monoclonal antibodies we have generated to bind these conjugate vaccines. Likewise the conjugate vaccines are able to inhibit the opsonophagocytic activity of these monoclonals. These vaccines will be used primarily to generate hyperimmune globulin for passive prophylaxis and treatment.

Series of mouse monoclonals against 12 of the 17 immunotypes of P. aeruginosa have been developed. These antibodies are directed against immunotype specific determinants located in the O-side chain and core regions. Several of the monoclonals demonstrate limited cross reactivity between immunotypes and several deep core monoclonals are broadly cross reactive. The monoclonals directed at immunotype specific determinants are opsonic with human complement and white blood cells and protect in mouse I.V. and I.P. challenge studies and in burned rats. They are able to prolong the time till death in neutropenic rat studies. Clearance studies have demonstrated rapid clearance of organisms in animals pretreated with monoclonals compared to animals tested with heterologous monoclonals.

Monoclonals have been produced against *E. coli* 018 LPS. Two of six were opsonic with human WBC and complement. All of those tested were protective in mouse intraperitoneal challenge, challenge of endotoxin resistant mice and in newborn rat studies. These LPS monoclonals protect against strains that contain the K1 capsule. Epidemiologically we have demonstrated that a cocktail of 11 monoclonals against different *E. coli* 0 serotypes would protect against 60% of *E. coli* sepsis.

Because of the potential requirement for human monoclonals to prevent sepsis a system for finding and production of human monoclonals has been developed. Using our mouse-human fusion systems we have produced 6 stable human monoclonals against Pneumococcal polysaccharide, 4 stable human monoclonals against the protective antigen of Anthrax and several stable lines secreting antibodies against Thyroglobulin and TSH reception. We therefore have the capability to produce human monoclonal lines.

To prevent colonization of the trachea in traumatized and wounded service personnel attempts to understand the mechanism of attachment have been made. We have demonstrated in collaboration with Reuben Raphal (University of Florida) that Pseudomonas pili inhibit this attachment of *P. aeruginosa* to injured tracheal epithelial cells. Pili inhibited homologous and heterologous strains, but antibody against pili only inhibited attachment of the strain from which the pili were isolated. These results suggest that there are a limited number of receptors for pili attachment but that pili contain an immunodominant variable determinant against which most antibody is directed. This antibody is not broadly cross reactive. Monoclonal antibodies have been prepared against purified pili which demonstrate broader cross reactivity. Their ability to inhibit attachment is being studied. Fragments of pili containing common regions have been obtained from Dr. W. Parynehyeh and monoclonals are being prepared against these fragments. These monoclonals will be used to determine if there is a common cross reactive epitope in *P. aeruginosa* pili that mediates attachment. Such monoclonals would be useful as probes for gene libraries and directly as throat gargles to prevent colonization. To further study the antigenic heterogeneity of P. aeruginosa pili several gene libraries have been prepared using a cosmid cloning vector. Attempts to sequence pili by cloning and by RNA sequencing techniques are underway.

Plans:

Monoclonal antibodies to J5 core determinants, E. coli 0 antigenic, P. aeruginosa will continue to be developed and tested in vitro and in animal models. We plan to assess the efficacy of polyvalent monoclonal sera in protection experiments and to evaluate whether monoclonal antibodies directed at different virulence factors of the same organism may be synergistic. In the next 2 years we also plan to examine the levels and longevity of J5 antibody in human volunteers following J5 vaccination. Since the capsular polysaccharides of K. pneumoniae are such potent immunogens and highly protective in animal experiments, we shall consider the feasibility of a polyvalent K. pneumoniae capsular vaccine for human phase I and 2 trials if there are a limited number of capsular types in human infection. Finally, work will continue on the development of human monoclonal antibodies.

Production of monoclonal antibodies will be pursued with the objective of developing a complete set of serotype specific antibodies for use in monitoring the serotype of meningococcal group B case strains and in studying the epidemiology of meningococcal disease in the Army. In addition, a complete set of antibodies specific for the group-specific capsular polysaccharides will be developed to serve as reagents for serogrouping meningococcal isolates. Emphasis will be placed on identification of common surface antigens. Common antigens identified will be studied to determine their potential for use as a group B meningococcal vaccine.

Examination of the immunochemical characterization of the serologic reactions of neisseria will continue as follows:

1. Separate and physico-chemically characterize the oligosaccharide moieties of gonococcal lipooligosaccharide.

2. Characterize serologically the oligosaccharide moieties of lipopolysaccharides of serum sensitive and serum resistant strains of gonococci.

3. Identify lipooligosaccharides functioning as serum sensitive epitopes and epitopes shared by serum resistant strains.

4. Select oligosaccharide or detoxified lipooligosaccharide antigens of gonococci with vaccine potential and develop procedures to couple such antigens to appropriate gonococcal outer membrane proteins to construct divalent vaccines which will protect against gonococcal disease.

5. Develop procedures to construct above as synthetic vaccines.

Detoxified LPS-protein conjugate vaccines from *E. coli* will be prepared and combined with Pseudomonas conjugate vaccines and Klebsiella polysaccharide to produce human hyperimmune globulin against these organisms. Cells from immunized volunteers will be utilized to produce human monoclonal antibodies against these lipopolysaccharides and polysaccharides.

Army wide hospital field trials of either human hyperimmuneglobulin, mouse monoclonal, and/or human monoclonal will be pursued.

Active immunization of soldiers with J-5 vaccine which induces broad cross-reactive protection against the endotoxin component of gram negative organisms will be attempted in an effort to determine the duration of efficacy. This will be evaluated by the potency of human sera following immunization in animal models and binding assays.

A pilus vaccine which does not contain the immunodominant hypervariable region but does contain this common binding region will be constructed by chemical or recombinant techniques. Trials of this vaccine and monoclonals that inhibit attachment will be performed on medical and surgical intensive care units.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OG 6763	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AC	204 WWGC
b. CONTRIBUTING				
c. XXXXXXXXXX	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Rickettsiae - Host Interactions in Pathogenesis of Disease

12 SUBJECT AREAS
0613 Mcirobiology 0603 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	Cont	DA	C. In-house

17 CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	308
85	2.0	299

19 RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of CD&I
b. ADDRESS (include zip code) Washington, D C 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, D C 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR HEDLUND, K W
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-2146
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Oaks, E V g. NAME OF ASSOCIATE INVESTIGATOR (if available) Eisemann, C S

22 KEYWORDS (Precede EACH with Security Classification Code) (U) Rickettsiae; (U) Biochemistry; (U) Structure - Function Relationship; (U) Antigenicity (U) Lab Animals; (U) Guinea Pigs; (U) RAM I

23 TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Isolate and characterize subcellular fractions of rickettsiae which have potential as experimental immunogens. Locate and identify the rickettsial surface antigens that affect virulence. Evaluate in mice the immunogenic potential of rickettsial fractions. Investigate alternate techniques to facilitate the early diagnosis of rickettsial diseases and detect rickettsial antigens. These studies will aid in improving the accurate rapid diagnosis of rickettsial infections and in the development of vaccines capable of protecting troops deployed in areas endemic for rickettsial diseases.

24. (U) Isolate subcellular fractions of rickettsial organisms using low ionic strength buffer, ether extraction, and genetic recombination methodologies. Characterize potential immunogens by physiochemical and immunological methodologies. Use antibody prepared against rickettsial surface antigens to evaluate the possible role of the antigens in body fluids of infected laboratory animals. Isolate and determine the biochemical and immunologic characteristics of these antigens.

25. (U) 83 10 - 84 09 Identification, characterization and purification of R. tsutsugamushi antigens requiring a high resolving capacity and high level of sensitivity have been approached successfully by combination of SDS polyacrylamide gel electrophoresis and electro-immunoblotting (western blotting). Twelve polypeptides in Karp strain were recognized with hyperimmune rabbit sera. Molecular weights range from 40,000 to 120,000 daltons. Monoclonal antibodies were reactive with 43K and 50K polypeptides. The serum of monkeys previously infected with Karp tested by western blotting showed four major polypeptide antigens, some individuals recognized addition unique antigens. After solubilization of rickettsiae with triton X-100, urea and beta-mercaptoethanol the major rickettsial antigen peaks were successfully separated by preparative isoelectric focusing. Work continues on the alternate DNA recombinant method of producing rickettsial antigens. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

Project 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 204 Rickettsiae-Host Interactions in Pathogenesis of Disease

Investigators:

Principals:
COL Kenneth W. Hedlund, MD
Edwin V. Oaks, PhD
Christine S. Eisemann, MS

Problems and Objectives:

Scrub typhus has been a recurrent problem for American troops both during World War II and in Southeast Asia. It has been estimated that six percent of U.S. troops in Viet Nam hospitalized for malaria were also suffering from scrub typhus. Attempts at the production of an effective vaccine have been hampered by the lack of knowledge about which of the rickettsial antigens are responsible for the protective response one sees in nature following infection that has been the basic problem. Our two fold approach has been to first produce intact rickettsiae in tissue culture systems, striving to optimize production and minimizing eucaryotic contamination and to use these well characterized plaque-purified rickettsiae as a source of immunogens for a potential vaccine. The second has been to produce rickettsial antigens by recombinant techniques. As previously noted the success of DNA recombinant techniques in other areas of medicine and industry makes the use of these methods an attractive alternative for the production of rickettsial antigens.

Progress

The identification, characterization and purification of R. tsutsugamushi antigens require procedures with high resolving capacity, a high level of sensitivity and the capacity to overcome the significant quantity of host-cell contamination in scrub typhus antigen preparation. To this end, SDS-polyacrylamide slab-gel electrophoresis coupled with electroimmunoblotting (Western blotting) procedures have been used to detect the major protein antigens of R. tsutsugamushi. Hyperimmune rabbit sera reacted with at least 12 polypeptide bands (4 major bands) in R. tsutsugamushi (Karp) infected in all lysates. The molecular weights of these antigens ranged from 40,000 to 120,000 daltons. Several of these major antigens were present in other scrub typhus strains (Kato and Gilliam) as well. Mouse monoclonal antibodies were reactive with Karp polypeptide antigens 50K and 43K. The electroimmunoblotting was also used to determine which polypeptide antigens were recognized by Karp-infected monkeys. All monkeys tested reacted with four major protein antigens but individual monkeys, in some cases, recognized unique polypeptide antigens. The availability of Western blotting analysis will allow the qualitative and quantitative detection of rickettsial antigens in preparations of various degrees of purity. In addition, this procedure provides a mechanism for monitoring the humoral response to specific protein antigens without the need for a purified antigen.

The production of sufficient quantities of rickettsial antigens by conventional techniques for the eventual use in vaccines is extremely limited by the low quantities of rickettsial antigen recovered from either tissue culture or yolk sac material. Therefore, recombinant DNA approaches are underway in order to produce rickettsial antigens in *E. coli*. However, it is not clear at this point exactly which of the rickettsial antigens are (1) recognized by infected humans and (2) are essential for protection in the human host. Therefore, it is essential to obtain purified antigen to determine which antigens are required for protection in available animal models. Once the protective antigen(s) are determined, the recombinant DNA effort can be focused on this defined set of antigens. We have been able to separate, into relatively pure fractions, the major polypeptide antigens of *R. tsutsugamushi* (Karp). Rickettsial proteins, solubilized from infected L-cells with a solution containing triton X-100, urea and β-mercaptoethanol, were applied to a preparative isoelectric focusing bed and separated based on net charge over a pH range of 4.0 to 9.5. Fractions were collected and analyzed by Western blotting procedures, using a hyperimmune rabbit serum as the probe. Major antigen peaks were found in narrow pH ranges consisting of a spread of 3-4 fractions. The major peaks were at (1) pH 5.5-5.85 (this peak reacted with monoclonal antibodies to the 43K antigen); (2) pH 6.3-6.7, molecular weight of approximatity 63,000 and (3) pH 7.6 to 7.8 with a molecular weight of 50,000. As the separation of major antigens is possible by preparative isoelectric focusing, this procedure will be used to generate sufficient quantities of relatively pure antigens to be used in pilot protection studies in mice. If it is found that these animals are protected by these isolated rickettsial antigens then the recombinant DNA effort will be focused on that antigen.

Recommendations:

At present, using tissue culture techniques coupled with immunoelectrofocusing, it is possible to isolate four peaks which contain the four predominant polypeptides found in scrub typhus rickettsia. These four bands have been serologically recognized by antibodies found in hyperimmune polyvalent rabbit sera as well as the sera of primates who have survived scrub typhus infections. Mouse monoclonal antibodies have also identified two of these polypeptides. It is our intention to use this immunoelectrofocusing technique to isolate rickettsial antigens and to evaluate the components either individually and/or in combination for their ability to elicit protective responses in animal models prior to rickettsial challenge.

The DNA recombinant effort we anticipate will receive strong impetus with the arrival of a molecular biologist. The recent rickettsial recombinant successes of Krause, Winkler and Wood with R. prowazekii heighen our confidence in the feasibility of this approach. Of course, the methods being used to identify and purify tissue culture propagated rickettsial antigens can also be applied to rickettsial antigens produced by recombinant techniques.

A joint venture undertaken with the Department of Rickettsial Diseases at Naval Medical Research Institute has resulted in large numbers of hybridoma fusions. Unfortunately, our ability to screen all of these clones, as rapidly as we would like, is limited by the availability of manpower. We are addressing this in the newest manpower survey and requesting additional technical support.

Publication:

Eisemann, Christine S., Nypaver, Matthew J., and Osterman, Joseph V. Susceptibility of inbred mice to rickettsiae of the spotted fever group. Infect. and Immun. 43:143-148, 1984.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 0A 6514
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUMRY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AD	205 WWG4
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code): (U) Vector Transmission of Militarily Important Infectious Diseases

12. SUBJECT AREAS: 0603 Biology 0613 Microbiology

13. START DATE: 65 07
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT/GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	6.0	657
85	5.0	703

19. RESPONSIBLE DOD ORGANIZATION
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g. NAME OF ASSOCIATE INVESTIGATOR (if available): Ware, R

21. GENERAL USE: FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Malaria; (U) Mosquitoes; (U) Trypanosomiasis; (U) Tsetse flies; (U) Leishmaniasis; (U) Sand flies (U) RAMI

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Develop physiological means of interrupting malaria and leishmaniasis transmission through an understanding of factors affecting parasite infectivity in vivo and in vitro. Refine models of malaria, leishmaniasis and African trypanosomiasis transmission to obtain large numbers of parasites for the study of immune mechanisms. Assess competence of closely related species as malaria and leishmaniasis vectors. Develop method of testing repellents against tsetse flies. Develop a field applicable serological test to detect anophelines infected with falciparum malaria. Realization of objectives may lead to prevention or control of malaria, leishmaniasis, and trypanosomiasis in military troops.

24. (U) Continue producing falciparum sporozoites needed for characterization of monoclonal antibodies. Identify factors that influence that infection process of falciparum malaria in anopheline mosquitoes. Establish colonies of phlebotomine sand flies for leishmaniasis transmission studies. Compare susceptibility of different sand fly and anopheline species to leishmanial and malarial parasites respectively. Develop the ELISA test to detect P. falciparum sporozoites in mosquitoes. Refine methods for testing repellents against tsetse flies. Produce large numbers of procyclic trypanosomes for African trypanosomiasis vaccine feasibility studies.

25 (U) 83 10 - 84 09 Upwards of 80 million P. falciparum sporozoites were isolated from anophelines infected with cultured parasites. Emphasis was on culturing the 7G8 clone of a Brazilian isolate. Field testing of both P. falciparum and P. vivax double antibody ELISAs was carried out with considerable success in both Thailand and Mexico. Laboratory-infected sand flies were used to identify the infective stage of Leishmania against which monoclonal antibodies are being generated for field and clinical use. The tsetse colony was self-sustaining and metacyclic forms have been provided to the Dept. of Immunology. For technical report see Walter Reed Army Institute of Research Annual Report 1 Oct 83 to 30 Sep 84.

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PROJECT 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND
HEALTH HAZARDS

WORK UNIT 205 Vector Transmission of Militarily Important Infectious Diseases

Investigators
Principal: Richard G. Andre, LTC, MSC

Associate: Donald R. Roberts, LTC, MSC; Peter V. Perkins, MAJ, MSC; Philip G. Lawyer, MAJ, MSC; Robert A. Wirtz, CPT, MSC; Ronald A. Ward, Ph.D.; Imogene Schneider, Ph.D.; Thomas R. Burkot, Ph.D. (NRC Fellow); Edgar D. Rowton, Ph.D. (NRC Fellow); David E. Hayes; Lawrence M. Macken; SP5 Megan G. Dowler; SP4 Lesyle Graves; SP4 Pedro Quintero; PFC Mark L. Haxton

Problems and Objectives

Malaria, trypanosomiasis and leishmaniasis are arthropod-borne diseases of great military medical importance. Consequently, the development of vaccines for these diseases is a high priority endeavor. The detection of protective antigens against the sporozoite stage of falciparum malaria is one of the most promising approaches to a malaria vaccine. Antigen variation at least in the tsetse fly transmitted forms of African trypanosomiasis and use of fly-form parasites for identifying protective antigens also offers the most promising approach to vaccine development. An effective vaccine must be active against the arthropod-borne form of parasite. Thus laboratory systems also are needed for producing infected vectors to challenge experimentally immunized animals against leishmaniasis, trypanosomiasis and malaria. The value of sand fly transmitted leishmanial parasites for detecting protective antigens is currently under study. Current research objectives are to 1) develop or improve on existing laboratory transmission systems for the groups of diseases listed above, 2) develop immunochemical tests for detecting infectious parasites in natural vector populations for malaria and leishmaniasis and 3) determine factors influencing infection rates of malaria, African trypanosomiasis and leishmaniasis parasites in anopheline, tsetse and sand fly vectors, respectively.

Progress

Malaria: Responsibility for culturing the blood stages of *Plasmodium falciparum* for infecting anopheline mosquitoes was assumed by the Entomology Department. As a consequence, production of mosquitoes for infective feeds could be geared more closely to gametocyte maturation in the cultures. This resulted in greater numbers of mosquitoes per infective feed than had been achieved in the past (1150 vs 665 ave). The percentage of infected mosquitoes per feed varied from 0 to 100% with oocyst numbers ranging between 1 and 200+ in the successful feeds. Approximately 82 million sporozoites were isolated during the year from the 7G8 clone of *P. falciparum*. These were apportioned for ELISA and CSP testing, monoclonal antibody production, RNA isolation, immunochemistry studies and IFA slides. Two other strains of falciparum malaria, T-4 from Thailand and LE5 from Africa, also were placed in culture. The T-4 strain produced much higher infections in mosquitoes (virtually 100%) than did the 7G8 clone but only for the initial cultures from frozen material. By the third or fourth subculture gametocytes, although still present in large numbers and morphologically mature in appearance, failed to infect any of the three anopheline species maintained in the departmental insectary.

Monoclonal antibodies developed against the sporozoites of *Plasmodium vivax* were used to develop an ELISA. Positive reactions were obtained only with homologous parasites when tested against sporozoites of various other species of *Plasmodium*. The vivax sandwich ELISA detected as few as 300-400 sporozoites per infected mosquito and was sensitive enough to detect one infected insect in a pool of 20 mosquitoes. Field trials of the vivax ELISA in southern Mexico confirmed that the monoclonal antibodies produced against Thailand sporozoites detected parasites from another geographic region. The initial field trials in Thailand of the falciparum ELISA indicated that antigenic diversity was present in the sporozoite population. Further characterization of the 22 monoclonal antibodies produced against falciparum sporozoites supports this finding with 2 monoclonal antibodies identified which identify different epitopes on the circumsporozoite protein coat. A falciparum monoclonal antibody has been identified which reacts with all sporozoites tested to date and an ELISA has been developed using this monoclonal. The question of antigenic diversity in sporozoite poopulations is being pursued because of its implication for the malaria vaccine development program.

Leishmaniasis: Laboratory colonies of the following sand fly species currently are maintained:

1. *Phlebotomus papatasi* (Israel)
2. *P. papatasi* (Egypt)

3. P. papatasi (India)
4. Lutzomyia anthophora (Texas, USA)
5. Lu. christophei (Dominican Republic)
6. Lu. diabolica (Texas, USA)
7. Lu. longipalpis (Brazil)
8. Lu. shannoni (Florida, USA)

Flies from these colonies are used in repellent testing and to produce sand fly forms of Leishmania parasites needed for several ongoing projects.

Research conducted in collaboration with the Laboratory of Parasitic Diseases, National Institutes of Health, demonstrated sequential development of promastigotes from a noninfective to an infective stage in culture and in the sand fly vector. The generation of an infective stage was found to be growth-cycle dependent and restricted to nondividing forms.

A membrane feeding system was developed to infect P. papatasi with L. donovani. Promastigotes removed from the sand flies were used to generate monoclonal antibodies against the transmission stage of the parasite. Monoclonal antibodies also were produced against sand fly forms of L. mexicana amazonensis. Research is in progress on the use of these monoclonal antibodies in enzyme-linked immunosorbent assays (ELISA) for rapid detection of infected sand flies in wild populations. Infected and noninfected sand flies also were used to test the efficacy of KDNA hybrid probes in another system for rapid detection of Leishmania-infected sand flies.

Eight standard topical repellents and a synthetic pyrethroid were evaluated against the Old World sand fly. P. papatasi, using a dose-response testing procedure on white rabbits. The sensitivity of P. papatasi to repellents is similar to that of the New World sand fly, Lu. longipalpis, and much higher than that of most mosquitoes.

African Trypanosomiasis: The tsetse colony achieved a maximum size of 8000 females in March 1984. Since then, the colony has been reduced to 5000 breeding females through the elimination of older, non-reproducing flies. Current production of pupae is in excess of 300 pupae daily which covers current requirements. Fifteen infective feeds were conducted to provide metacyclic stage parasites for immunological studies with the Department of Immunology. From these feeds, 62 flies, or 5% of those given an infective meal had salivary gland infections. This infection rate is lower than anticipated and studies are being conducted to determine the factor(s) responsible for this change in infectivity. Studies on the effect of tsetse digestive enzymes on midgut infection rates were completed. The differences noted, although statistically significant, do not indicate a need to change current infection practices.

Five standard topical repellents and a synthetic pyrethroid were evaluated for use against the tsetse fly, using a dose-response procedure on white rabbits. In decreasing order of repellent effectiveness was dimethyl phthalate, 2-ethyl-1,3-hexanediol, permethrin, N,N-diethyl-m-toluamide, Inalone and Citroynl based on 0-hr ED50 tests. Permethrin exhibited both insecticidal and repellent activity at the concentrations used.

Recommendations for the future

The production of malaria sporozoites should be continued in support of the sporozoite vaccine development effort. Emphasis should be placed of selecting and properly managing malaria clones that produce high infection rates and large numbers of sporozoites.

Population selection studies should be conducted to develop strains of mosquitoes and parasites that produce maximum numbers of sporozoites. As part of this work studies should be performed to identify the barriers to sporozoite production.

Attempt modification of the falciparum and vivax ELISA assay for simplification of the test procedures and substitution of less costly, more readily available reagents while maintaining the sensitivity and specificity of the assay. Conduct field tests as necessary to evaluate the specificity of the assay, to address the problem of antigenic variation in different geographic areas and to use the ELISAS as research tools in studies on the epidemiology of human malaria. The Department of Entomology is receiving currently dried mosquitoes from several different geographic areas for testing.

Develop systems for producing large numbers of the various strains (species/subspecies) of leishmanial parasites within the sand fly and develop ELISA's for the fly forms. Produce monoclonal antibodies against the infective form of the leishmanial parasite. Compare infectivity of infective forms versus culture forms for vaccine feasibility studies.

Continue to improve the tsetse fly colony, conduct *in vitro* colony maintenance studies and increase the yield of tsetse fly-form parasites.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OA 6436	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102 BS10	AE	206 WWGF
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(U) Microbial Genetics and Taxonomy

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13. START DATE 63 08	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

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FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	4.0	748
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19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of Communicable Disease & Immunology Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR BARON, L S
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-2230
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23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23 (U) To define in genetic and molecular terms gene transfer, antigenicity, and virulence of pathogenic enteric bacteria which because of their disease producing capabilities are of importance to military medicine. The ultimate aims are prevention and improved treatment of enteric infections of Army personnel. A major approach is to modify enteric bacteria genetically to produce any desired antigenic structure and level of pathogenicity. Such strains can serve as vaccine strains or as tools to study the infectious process. Specific DNA probes are identified to serve as rapid detection tools.

24 (U) Standard genetic, molecular genetic and recombinant techniques are used for strain construction and modification. Biochemical, cell culture and animal assays are employed to assess virulence properties.

25 (U) 83 10 - 84 09 A genetic transposition system has been set up in Shigella so that chromosomal virulence genes can be isolated without knowing their actual chromosomal location. The genes responsible for attachment of Shigella to epithelial cells have been cloned via genetic engineering methods and are being characterized. The genes encoding the Vi capsular antigen of Citrobacter freundii have been cloned and analyzed. A specific 8.6 kilobase pair fragment has been shown to act as a highly specific DNA probe for the detection of S. typhi, the causative agent of typhoid fever. Probes have been developed that are specific for several species of Leishmania, that cause disease in Central and South America. Similar DNA probes specific for Shigella have been isolated and are being assessed for specificity. S. typhi Ty21a oral vaccine derivatives expressing various Shigella antigens are being constructed. For technical report see Walter Reed Army Institute of Research Annual Report 1 Oct 83 - 30 Sep 84.

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PROJECT 3M161102BS10 RESEARCH ON MILITARY DISEASES, INJURY AND HEALTH HAZARDS

Work Unit 206: Microbial Genetics and Taxonomy

Investigators:

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Assistant: F.A. Rubin, Ph.D.

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Problem

Bacterial infections of the gastrointestinal tract have always been a serious health hazard to those entering an area where modern sanitary practices and facilities are lacking. More than 50% of the military personnel involved in field operations frequently are incapacitated by enteric bacterial illnesses. These enteric organisms can produce severe stomach cramps, nausea, vomiting, intestinal ulcerations, bacteremia, dysentery and diarrhea. Such enteric bacterial infections generally occur within several days after personnel enter an area where sanitary conditions are deficient or disrupted. Effective prophylactic field measures do not exist for many of the severe enteric disease agents e.g., Shigella, Salmonella, enterotoxigenic E. coli. Since native populations do develop an immunity to the enteric organisms normally indigenous to their environment, the development and use of effective enteric vaccines should act to augment the level of natural immunity, thus reducing the inherent disease level in these areas. Also, effective enteric vaccines would stimulate immunity in military troops who frequently are highly susceptible individuals. Current broad objectives within this work unit include the development of mono-and multi-valent vaccines against enteric organisms, testing vaccine efficacy in animal models, and using molecular and genetic approaches to study the mechanism of disease pathogenesis resulting in pertinent information that can lead to the development of suitable techniques for the construction of improved vaccines or for the rapid detection and treatment of pathogenic bacteria in a diseased patient. Towards this end, a variety of basic investigations into the molecular nature of bacterial gene expression and intercellular gene transfer, employing classical and new molecular genetic procedures including recombinant DNA technology, are conducted.

Whole animal and cell culture systems are employed to assess bacterial virulence traits and vaccine efficacy.

Progress

Our research studies, conducted in collaboration with the Dept. Bacterial Diseases, have resulted in the conclusion that large plasmids (120-140 megadaltons in size) are necessary for the virulence of all strains of bacteria that cause dysentery (i.e., all *Shigella* species and certain *Escherichia coli* strains; see ref. 1, 2). Specifically, we have shown that all virulent *Shigella sonnei* strains harbor a 120 Mdal plasmid that encodes the major protective cell surface antigen, the form I somatic antigen, of this species (3, 4). Also, virulent isolates of all six serotypes of *S. flexneri* have been found to carry a 140 megadalton plasmid (5), as do dysenteric strains of *E. coli*. Animal and tissue culture assays have revealed that these large plasmids encode some virulence property that allows these virulent bacteria to invade epithelial cells, which is a primary step in the disease process of dysentery.

Genetic engineering procedures have been developed to isolate both plasmid-mediated and chromosomally-borne virulence genes of *Shigella*. Very recently, procedures for transposing *Shigella* virulence genes to plasmids via Mu virus-mediated transposition have been developed. Several *Shigella* chromosomal genetic loci have been cloned in this manner to isolate the *kcpA* gene virulence locus. Biochemical studies will be initiated to determine the products of the *kcpA* locus. Using recombinant DNA procedures, the plasmid genes determining form I O-antigen synthesis have been subcloned on a 5 kilobase pair DNA fragment. This cloned, antigen-expressing fragment was inserted into form II *S. sonnei* recipient cells where it restored form I antigen expression but not virulence, as expected; the plasmid genes controlling epithelial cell invasion are still missing from this strain. In related studies, all fragments of the large 120 and 140 Mdal virulence plasmids have been cloned onto cosmid vectors. No single clone contains all of the genes necessary for epithelial cell invasion but one group of recombinant plasmids encodes the first step of disease pathogenesis, epithelial cell attachment. These adherence functions are now being analyzed in further detail. Recently, new tissue culture systems have been established to aid in the identification of recombinant clones that carry various genes involved in epithelial cell attachment/invasion. Cloned *Shigella* antigens will be used to probe the pathogenic mechanisms involved in dysentery, to construct vaccine strains, and to develop DNA probes for disease diagnosis.

The galactose-epimeraseless *S. typhi* Ty21a oral vaccine strain of Germanier and coworkers (6) appears to be a potential carrier of many antigenic determinants and should prove useful in constructing multivalent oral vaccines protective against many intestinally acquired diseases (7,8,9). A Ty21a genetic derivative carrying the genes for and expressing the *S. sonnei* form I antigen has been constructed (7). This candidate oral vaccine protected mice against challenge by virulent *S. typhi* and *S. sonnei* challenges (7) and has been shown to stimulate rabbit intestinal IgA production (8). Recent volunteer studies indicate that the vaccine is safe at doses of 10^{10} cells and causes no adverse effects; and challenge of immunized humans with live *S. sonnei* cells under controlled conditions resulted in a vaccine efficacy of > 60%. This vaccine is going to be tested in a field trial in early 1985. Chemical analyses of the LPS of this hybrid vaccine have shown that polymerized form I O-antigen is expressed on the cell surface but it is not covalently linked to *S. typhi* core oligosaccharide (10). This type of hybrid may constitute a better oral vaccine because the O-antigen may be able to stimulate higher levels of IgA; further studies are planned to follow up this observation. A second oral vaccine derivative that expresses the *S. flexneri* 2a O-antigen has been developed and was found to protect mice against homologous challenge organisms. Chemical studies of the LPS of this hybrid suggest that the *S. typhi* 9, 12 antigens are not expressed; more derivatives are being constructed to overcome this problem. Finally, Ty21a genetic carriers that express the antigenic determinants of other predominant causes of enteric disease (e.g. *S. flexneri* and *S. dysenteriae* strains, dysenteric *E. coli*) are being developed. It appears that 4 oral vaccines protective against *S. sonnei*, *S. flexneri* 2a and 3, and *S. dysenteriae* 1 could prevent greater than 95% of the shigellosis encountered worldwide. Finally, attenuated *Shigella* strains containing *galE* and other mutations are being developed to assess their value as alternate oral vaccines.

Plasmids have recently been implicated in the virulence of *Salmonella* species. We conducted a large scale plasmid analysis of *S. typhi* strains isolated worldwide and have determined that plasmids are not generally involved in the virulence of this species. However, a plasmid of *S. typhimurium* has been reported to encode essential virulence traits. Our present studies are aimed at phenotypically tagging this plasmid, cloning all DNA regions, and analyzing the virulence components. In separate studies, the genes encoding the virulence capsular antigen of *S. typhi* have been cloned and subcloned. Genetic and biochemical analyses of this cloned region to determine the number of genes involved, the enzymatic functions, and the sequences controlling gene expression have been initiated. An 8 kilobase pair DNA fragment has been shown to act as a highly specific DNA probe for the detection of *S. typhi* (11). This probe is being field tested in the Far East, in Chile, and in Peru to assess its value as a diagnostic aid in detecting *S. typhi*. Eventually, a suitable kit will be assembled for rapid detection (i.e. within 2-3 hrs.) of *S. typhi*, to be used by deployed troops.

Collaborative studies have been initiated to clone genes of *Neisseria*, *Pseudomonas*, and *Rickettsia*. The genes encoding *Neisseria gonorrhea* pili have been cosmid cloned and appear to be expressed on the surface of recipient *E. coli* cells. Electron microscopic and immunochemical analysis of this GC pili expression in *E. coli* is in progress. Systems have been developed to cosmid clone in *E. coli* various *Pseudomonas* genes which can then be shuttled back to *Pseudomonas* for detection of gene expression. Studies to clone *Pseudomonas* pili are in progress. The rickettsial gene encoding citrate synthase has been cloned from *R. tsutsugamushi* and expressed in *E. coli*; and a concerted search in being made to detect cloned rickettsial antigen genes in *E. coli*. All of these collaborative efforts are aimed at isolating surface antigen genes to be used in vaccine development and to characterize the pathogenic mechanisms of disease.

A number of strains of *Leishmania* which are responsible for the occurrence of visceral disease in different parts of the world have been investigated. Examination of the restriction enzyme fragment patterns of the kDNA from these parasites shows that the fragment patterns of the kDNA of strains isolated from different parts of the world have several distinct fragments which are unique for each strain. While this method of analysis is complex and cannot easily be performed on a diagnostic basis, it has been possible to isolate these distinct fragments and they are being tested for use as specific DNA probes for rapid differentiation of *Leishmania* species.

Recommendations for the Future:

1. The recombinant cosmid clones of the *Shigella* virulence plasmids will be analyzed to determine clones expressing epithelial cell penetration ability. Recombinant plasmids coding for adherence and cell invasion will be analyzed biochemically to determine the proteins involved. The virulence components will be evaluated for antigenic potential for vaccine development.

2. Cloned DNA fragments of *Shigella* will be assessed for their ability to act as species specific and general hybridization

probes for use in rapid diagnostic detection techniques. Kits will be developed for field use by deployed troops.

3. The Vi antigen DNA probe for *S. typhi* detection will be evaluated under field conditions in collaboration with labs in Thailand, Chile, and Peru. Use of non-radioactive DNA-labelling techniques will be assessed.

4. Genetically modify the *S. typhi* Ty21a oral vaccine carrier strain to express the antigenic determinants of *S. flexneri* 3 and *S. dysenteriae* 1 strains. These new strains could be used in combination with previously developed *Shigella-S. typhi* hybrid vaccine strains to produce a single multivalent vaccine to protect against typhoid fever and the 4 predominant causes of shigellosis. In addition, the Ty21a carrier strain will be modified by adding other antigenic determinants of *Shigella* and other enteric pathogens.

5. Use genetic engineering and classical mutation studies of *Shigella* and *Salmonella* to dissect the steps involved in disease pathogenesis. Cloned virulence genes will be analyzed biochemically and immunologically. This basic genetic and biochemical information should define new steps in the pathogenic process and give new insights into methods for both prophylactic and chemotherapeutic intervention.

6. Tagged *Salmonella* virulence plasmids will be analyzed in various enteric bacteria, using several virulence assay systems, to assess plasmid-borne virulence traits. These genes will be cloned via recombinant DNA procedures and analyzed biochemically. DNA fragments will be assessed as DNA probes for rapid identification of *S. typhimurium*.

7. New genetic studies, directed at creating mutations in various enteric disease agents (e.g. *Aeromonas*, enteropathogenic *E. coli*, etc.) will be conducted to begin a genetic analysis of virulence in these organisms. Mapped antigenic traits can be used in future vaccine development efforts.

8. Continue collaborative projects on cloning of *Neisseria*, *Pseudomonas*, and *Rickettsia* genes for the development of vaccines. Attempts will be made to isolate several rickettsial metabolic and virulence genes.

9. Collaboratively develop DNA probes and hybridization conditions for rapid detection and speciation of *Leishmania*. The probes should be sensitive enough to detect

Leishmania parasites in any clinical specimen or in infected sandflies.

10. In new vaccine efforts, attenuated mutants of *Shigella* will be developed and assessed as candidate oral anti-dysentery vaccines.

11. Recombinant plasmids encoding the *S. typhi* Vi capsular antigen will be genetically dissected to determine the number of genes involved and the sequences controlling the unique reversible expression of this antigen. Studies on the biochemical functions of each gene will also be initiated.

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(By L.S. Baron)

Feb 23, 1984	Typhoid-Shigella hybrid Oral Vaccine-Field Trial Plans. Briefing to Israeli Defense Forces, Tel Aviv. Israel
Feb 28, 1984	Oral Vaccine Production. Swiss Serum Institute, Berne, Switzerland
Mar 6, 1984	Construction of a Fused Plasmid Specifying *Shigella flexneri* 2a Antigens and its Transfer to the *Salmonella typhi* Ty2la Oral Vaccine Strain. L.S. Baron*, D.J.Kopecko, S.B. Formal, P. Guerry, L. Hale, R. Seid, O. Washington, and C.A. Life, Annual Meeting of American Society for Microbiology, St. Louis, MO

(By J.N. Coulby)	
Mar 6, 1984	Extrachromosomal Conservation of *Escherichia coli* Chromosomal DNA in Diploid Hybrids of *Salmonella typhi*. J.N. Coulby*, N.J. Snellings, K.F. Noon, J.A. Wohlhieter, E.M. Johnson, and L.B. Baron. Annual Meeting of American Society for Microbiology, St. Louis, MO
(By F.A. Rubin)	
Mar 8, 1984	Development of a DNA Hybridization Diagnostic Detection System for *Salmonella typhi*. F.A. Rubin* L.S. Baron, K.F. Noon, and D.J. Kopecko. Annual Meeting of American Society for Microbiology, St. Louis, MO
(By D.J. Kopecko)	
Oct 83 - Sep 84	Lecturer in semester course on the Biology of Bacterial Plasmids, FAES Graduate School at NIH, Bethesda, MD
Nov 18, 1983	Involvement of plasmids in *Shigella* virulence: vaccine implications. Dept. Microbiol. seminar, Univ. Maryland, College Park, MD
Dec 15, 1983	Genetic analysis of enteric bacterial virulence properties: application to disease diagnosis and antidiarrhea/dysentery vaccines. Symposium on Recombinant DNA Technology-Prokaryotes, XV Intern. Cong. Genetics, New Delhi, India.
Dec 19, 1983	Molecular genetic studies of *Shigella* virulence-vaccine applications. Symposium entitled "Recent Advances in Genetic Engineering," Indian Inst. of Chem. Biology, Calcutta, India.
Feb 7, 1984	Genetic studies of *Shigella* virulence; oral vaccine applications. Two seminars at Mayo Clinic and Mayo Graduate School, Dept. Cell Biol., Rochester, MN.

Feb 16, 1984	Molecular genetic analyses of *Shigella* virulence properties. National Institute of Dental Research, Bethesda, MD.
Feb 23, 1984	*Shigella*-an overview. WRAIR Briefing for Richard B. Lewis II, Director of Army Research and Technology, WRAIR, Washington, D.C.
Mar 11, 1984	Current Biology of Bacterial Plasmids-Application to Diagnosis, Treatment, and Vaccine Intervention. Society of Armed Forces Medical Laboratory Sciences Annual Meeting, Washington, D.C.
Mar 20, 1984	Plasmids and Gene Manipulations. Class for Military Medical Science Fellowship course in Microbiology and Immunology. WRAIR, Washington, D.C.
Apr 12, 1984	The Genetic Analysis of Virulence in *Shigella*: potential oral anti-dysentery vaccines. University of South Alabama, Mobile, AL.
May 15, 1984	Genetic determinants of *Shigella* virulence: oral vaccine applications. Univ. Alberta-University of Calgary Conference on Infection Diseases, Alberta, Canada.
May 21, 1984	Genetic analyses of virulence in *Shigella*: oral vaccine applications. Dept. of Microbiology and Immunology, University of Illinois, Chicago, IL.
Jun 20, 1984	A system for constructing candidate multipurpose oral vaccine to protect against Enterial Bacterial Diseases. Army Science Conference, West Point, NY.
Jul 18, 1984	Enteric Disease Research in the Army. Briefing for U.S. Army Program for Biotechnology Applications, WRAIR, Washington, D.C.
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Patent Application Pending:

Formal, S.B., L.S. Baron, and D.J. Kopecko. U.S. Patent Application #289,013, entitled "Oral Vaccine for Immunization Against Enteric Disease."

Kopecko, D.J., L.S. Baron, and F.A. Rubin. U.S. Patent Application submitted October 1983, entitled "A method for the Rapid Detection of Typhoid Fever Bacteria."

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 0A 6445	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM
83 10 01	D Change	U	U		CX	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AE	207 WWG3
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 82/83-62/3			

11. TITLE (Precede with Security Classification Code)
(U) Pathogenesis of Enteric Diseases

12. SUBJECT AREAS
0603 Biology 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
59 05	Cont	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	395
85	2.0	429

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of CD&I
b. ADDRESS (include zip code) Washington, D C 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, D C 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Hale, T L
d. TELEPHONE NUMBER (include area code) (202)-576 3551	d. TELEPHONE NUMBER (include area code) (202)-576 3344
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Formal, S B g. NAME OF ASSOCIATE INVESTIGATOR (if available) Washington, O

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Lab animals; (U) Diarrhea; (U) Bacillary; (U) RamI; (U) Salmonellosis; (U) Immunity; (U) Immunization; (U) Plasmids; (U) Genetics; (U) Mice; (U) Rats

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23(U) The pathogenesis of bacterial infections of the gastrointestinal tract is being studied to establish factors and mechanisms by which disease is provoked. Through an elucidation of such elements, procedures for prevention and control of diarrheal diseases can be devised. Diarrhea is a significant problem in military personnel operating overseas.

24(U) The genetic control of O-antigen specificity of enteric pathogens is being studied since such cell envelope components are of importance in disease and its prevention through vaccination. Interactions of bacterial pathogens and epithelial cells, especially mechanims of penetration are investigated. Attentuated living vaccines are developed.

25(U) 83 10 - 84 09 Six protein products of Shigella flexneri virulence plasmids were identified, and polyclonal rabbit antisera were raised against three of these proteins. Immunoblots using serum from Rhesus monkeys which had been challenged with S. flexneri showed that four polypeptides encoded by the virulence plasmid are the immunodominant antigens recognized after a shigella infection. Genes encoding four immunodominant plasmid-coded polypeptides were localized on a 40 kb region of the S. flexneri virulence plasmid, and expression of these genes was studied in avirulent mutants with transposon insertions. Expression of S. dysenteriae 1 somatic antigen in an Escherichia coli K12 hybrid strain was analyzed biochemically. (For technical report see Walter Reed Army Institute of Research Progress Report, 1 Oct 83 - 30 Sep 84).

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EDITION OF MAR 68 IS OBSOLETE.

Project 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 207 Pathogenesis of Enteric Diseases

Investigators:

Principal: Thomas L. Hale, Ph.D.

Associates: Samuel B. Formal, Ph.D.
Othello Washington, B.S.

Problem

Diarrheal disease has been a component of military campaigns since biblical times. In recent history these diseases played an important role in the British defeat at Gallipoli and caused significant illness in American troops in North Africa and the South Pacific in WWII, in Korea, in Lebanon and in Viet Nam. The pathogenesis of bacterial infections of the intestinal tract is studied using techniques of biochemistry, genetics, molecular biology, physiology and pathology to establish the factors and mechanisms which are involved in the disease process. The current objectives of this work are to understand the interaction of enteric pathogens with intestinal epithelial cells and to develop vaccines to prevent disease.

Progress

The invasive phenotype in *Shigella* sp. and enteroinvasive *Escherichia coli* is associated with the presence of a 140 Mdal plasmid. Expression of this plasmid was studied by radiolabeling newly synthesized polypeptides in minicells containing only plasmid DNA. It was found that the 140 Mdal plasmid encodes at least fifteen polypeptides. A two-dimensional electrophoresis system was devised to identify polypeptides which are unique to the enteroinvasive phenotype. Seven unique polypeptides were identified in *S. flexneri* serotypes 2a and 5 and in an enteroinvasive strain of *E. coli*.

Enriched fractions of these polypeptides were isolated by preparative isoelectric focusing, and polyclonal rabbit antiserum was raised against three of the seven plasmid-coded polypeptides. It was found that these antisera recognized virulent strains of *S. sonnei*, *S. dysenteriae* I, *S. flexneri*, and enteroinvasive *E. coli*. Since noninvasive strains of these species did not react with antiserum against plasmid-coded polypeptides, the association of these polypeptides with the invasive phenotype was confirmed. These plasmid-coded polypeptides are important antigens to be included in shigella vaccines.

Expression of *S. dysenteriae* I somatic antigen has been analyzed in *E. coli* K-12 hybrids harboring a 6 Mdal plasmid and the *his*$^+$ chromosomal region from the latter species. The plasmid alone modified the *E. coli* K-12 lipid A-core structure in a way which made it slightly heavier and antigenically unique. The addition of the *his*$^+$ chromosomal region allowed the expression of somatic antigen which was electrophoretically, antigenically, and chemically identical to wild-type *S. dysenteriae* I O-antigen. With this information an *E. coli* K-12 hybrid vaccine expressing Shiga somatic antigen can be constructed.

Future studies

1. Plasmid-coded polypeptides from virulent and avirulent *S. flexneri* will continue to be analyzed in the two-dimensional electrophoresis system with particular emphasis on investigating extracellular products in addition to cell-associated polypeptides.

2. The immune response to plasmid-coded polypeptides will be investigated in guinea pigs and monkeys.

3. An organ culture system will be developed for *in vitro* study of the invasion of the monkey colonic epithelium by shigellae.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA OC 6435	84 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY	61102A	3M161102BS10	AF	208	WWG8
b. CONTRIBUTING					
c. XXXXXXXXXX	STOG 82/83 —	6.2/3			

11. TITLE *(Precede with Security Classification Code).*
(U) Immunity in Protozoan Diseases

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
74 07	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		84	7.0	613
c. TYPE	d. AMOUNT			
e. KIND OF AWARD	f. CUM/TOTAL	85	6.0	568

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division CD&I
b. ADDRESS *(include zip code)* Washington, DC 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Hockmeyer, W T
d. TELEPHONE NUMBER *(include area code)* (202)- 576-3551	d. TELEPHONE NUMBER *(include area code)* (202)- 576-3544
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Haynes, J D g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Lab Animals; (U) Mice; (U) RAMI;(U) Antigens; (U) Protozoa; (U) Immunity; (U) Tropical Medicine; (U) Malaria

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23 (U) To conduct immunological studies of protozoan diseases with emphasis on malaria, to produce P. falciparum malaria antigens by in vitro techniques for immunoassay and immunochemical analysis. These studies will aid in the development of a vaccine to protect soldiers stationed in many areas of the world against a major military disease.

24 (U) The approach used in these studies is to study, in both animal models and through the use of in vitro techniques, the response elicited by the immune system, to determine the role of cellular and molecular mediators in these processes, and to design experimental immunogens which will provide the basis for future vaccine development programs.

25 (U) 83 10-84 09. Several vaccine candidate antigens have been identified. Six are schizont - merozoite surface antigens to which we have monoclonal antibodies recognizing at least 12 epitopes. These monoclonal antibodies are being used in an RIA to quantitate epitope-specific antibodies in immune inhibitory sera. Additional antigens are being studied which are released at the time of reinvasion and bind to red cells. Studies are underway using affinity purified antigens to vaccinate experimental animals in order to provide sera for screening recombinant DNA expression libraries, as well as for in vitro immune assays. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83 - 30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

PROJECT: 3M161102BS10 RESEARCH ON MILITARY DISEASE,
INJURY AND HEALTH HAZARDS

Work Unit 208 Immunity in Protozoan Diseases

Investigators:

Principals:	LTC J. David Haynes, MC COL Carter L. Diggs, MC LTC Wayne T. Hockmeyer, MSC
Associates:	Dr. Jeffrey A. Lyon LTC(P) Jeffrey D. Chulay, MC MAJ Terence J. Hadley, MC CPT James L. Weber, MSC CPT James E. Egan, MC Ms. Christine Eisemann Ms. Teresa Reaud-Jareed Sp5 Lisandro Reyes Sp4 Dale Edwards

Problems and Objectives:

Malaria has caused many lost man hours in every major military conflict. Protecting soldiers in the field by administering one or more immunizing malaria vaccine doses before deployment would be logistically much simpler than currently available methods of controlling mosquito populations or administering antimalarial drugs weekly in the field. We are investigating immune responses to malaria blood stage antigens and are beginning to produce candidate vaccine antigens by genetic engineering.

The major objectives are (1) to identify the best parasite antigens to further evaluate as vaccine candidates, (2) to determine how much antigenic variation there is, and (3) to produce the antigens through genetic engineering in bacteria in sufficient quantity and purity so that then (4) they can be tested for their ability to induce protective immunity in experimental animals and (ultimately) people.

Progress:

We have extended our research using inhibitory immune sera, monoclonal antibodies (MAb) and protease inhibitors, and have identified 11 schizont-merozoite surface antigens which are good

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candidates for vaccine development (1). We have produced MAb against most of these. In some cases we have several MAb to different epitopes on the same antigen, including antigens with molecular weights of 195kd, 115kd, 101kd, 83kd, 65kd, 50kd, 45kd, 34kd, and 15kd (2).

One MAb (3D3) against the 83kd merozoite surface antigen is particularly valuable since it seems to recognize a repeating determinant and thus should be able to recognize a recombinant DNA fusion protein during the screening of an expression library. MAb 3D3 strongly reacts with denatured 83kd antigen blotted onto nitrocellulose filters, and it reacts in a 2-site radioimmunoassay (RIA) designed to distinguish repeating from single epitopes. Collaborations with Dr. R. Howard at NIH and Dr. J. McBride in Edinburgh confirmed our finding that this MAb 3D3 recognizes a new serotype-specific determinant on the 195kd major surface glycoprotein (and its 83kd processed product). This places us in a good position to be first to identify this 195kd antigen in a recombinant DNA expression library since there has been more success screening with MAb to repeating epitopes (3) than with MAb to single epitopes. As yet no one else seems to have a similar MAb. Human inhibitory sera from two sources immunoprecipitate the 83kd antigen (4,5). Strain-specific inhibitory human sera (6) have antibodies to the epitopes recognized by MAb 3D3 and another MAb against the 83kd antigen as determined by a competitive RIA (5).

Two dimensional electrophoresis demonstrated that this 83kd antigen, which is stabilized by inhibitory antibody, is the same 83kd antigen which is stabilized by certain protease inhibitors, though it is normally rapidly lost from the merozoite surface after processing from its 195kd precursor at the time of schizont rupture (Lyon, in preparation).

We now have the ability to isolate intact genes from parasite genomic DNA using mung bean nuclease and to clone them into the lambda gt11 expression system, as was done for the gene encoding the major surface antigen of P. falciparum sporozoites (7,3). We will soon use our antibodies to screen libraries made with this advanced cloning technique.

Three of the MAb have been used to affinity purify the corresponding 83kd antigen which has then been used to immunize rabbits and produce monospecific antisera. Certain methods of preparing and eluting the 83kd antigen from MAb columns resulted in better purity and antigenicity than did other methods (8). The ability to increase purity related to other studies which showed that several of these antigens (particularly the processed products of the 195kd, including the 83kd antigen) were associated as an antigen complex which could be dissociated when antigen preparations were treated with p-chloromecuribenzoate. MAb are also being used to purify other antigens.

Several parasite antigens (including 220kd, 170kd, 115kd, 83kd, 73kd, 65kd, 45kd, 40kd, and 29kd) have been identified in culture supernatant fluids (4,8). (Dr. D. Camus is a visiting professor working in our section during the past year). One of these antigens (170kd) binds to normal human red cells which can be invaded by *P. falciparum*, but does not bind to a variety of red cells which are not invaded. Thus this parasite component exhibits some of the characteristics of a *P. falciparum* merozoite receptor necessary for invasion. The 170kd antigen was recognized by human immune sera after binding to red cells. Affinity purified 170kd antigen has been used to immunize rabbits in order to produce monospecific antibody.

A 115kd antigen of *P. falciparum* was identified which was recognized by sera from animals immunized with the 140kd protective surface antigen of *P. knowlesi* (9, 10). More recently it was determined that the *P. falciparum* 115kd may not be directly homologous to the *P. knowlesi* 140kd antigen. However, we are continuing to pursue it, since (a) the 115kd antigen seems to be on the surface of the *P. falciparum* merozoite, (b) it is identified by inhibitory human sera (see above), and (c) we have identified 3 MAb against it. The MAb will be used to affinity purify the antigen and produce antibodies which will be more likely to recognize a recombinant fusion protein in screening a gt11 expression library. Similar efforts are underway with the 101kd surface antigen (which may have a 220kd precursor).

As well as screening recombinant DNA expression libraries, all of the above antibodies are being tested for inhibition in a recently improved rocking suspension invasion inhibition assay. Some of them will be used for immunoelectron microscopic localization of the corresponding antigens.

Dr. Lyon has produced more exciting MAb than our small group is able to work up, and we have been happy to enter collaborations with several other groups. One has already resulted in a publication (11). Ongoing collaborations include (a) with Dr. R. Dayal of WHO, Geneva, development of an ELISA to detect circulating parasites, (b) with Dr. J. McBride, U. of Edinburgh and Dr. G. Campbell, CDC, studies of serotype specificity, and (c) with Dr. R. Howard, NIH, studies of surface antigens on infected red cells.

Additional advances have included the improved definition of the amino acid, vitamin, and osmolarity requirements for parasite culture, which will help us to better produce parasite antigens and analyse growth inhibition. Studies which may have implications for cell mediated immunity included the development, cloning, and study of a modified parasite resistant to sorbitol lysis; and the demonstration that human polyamine oxidase can inhibit the growth of *P. falciparum*.

Although we are primarily concerned with the development of a vaccine against blood stage malaria parasites, our group made several key contributions to the recent cloning of the sporozoite surface antigen (3) which include: (a) developing crucial aspects of the immunoscreening assay which was then used to detect the recombinant clones expressing the sporozoite CSP antigen, (b) helping to work out key aspects of the ELISA assay and western blotting analysis of the CSP antigen.

Recommendations:

Continue with present research plans. We need to (1) recruit another recombinant DNA scientist; (2) further explore the possibilities for coordinating in-house efforts with other groups and companies through collaborations and contracts: (3) acquire two research assistants to help the three professional staff who now have not technicians; (4) renovate some of the labs and add two well equipped chemistry labs with chemical fume hoods: (5) continue to expand our ability to increase productivity using microcomputers and the VAX; (6) plan for large increases in manpower and budget which will be needed to test vaccine candidate antigens as they are produced in the next few years.

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Presentations and Abstracts:

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2. Camus D., Lyon J., Reaud-Jareed T., Haynes D., and Diggs C.L. 1984b. Merozoite surface antigens of *P. falciparum* purified from culture supernatant fluids using monoclonal antibodies. Joint Mtg. Am. Soc. Trop. Med. Hyg. and Roy. Soc. Trop. Med. Hyg., Baltimore, M.D.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OC 6444
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AH	210 WWH2
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Biochemical Research on Military Diseases

12. SUBJECT AREAS
0601 Biochemistry 0613 Microbiology

13. START DATE: 76 07
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	3.0	652
85	5.0	703

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code): Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL: Top, F H Jr
d. TELEPHONE NUMBER (include area code): (202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
Division of Biochemistry
b. ADDRESS: Washington, D.C. 20307- 5100
c. NAME OF PRINCIPAL INVESTIGATOR: Gemski, P
d. TELEPHONE NUMBER (include area code): (202)-576-2594
f. NAME OF ASSOCIATE INVESTIGATOR (if available): Brown, J E
g. NAME OF ASSOCIATE INVESTIGATOR (if available):

21. GENERAL USE: FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) lab animals; (U) rabbits; (U) RAMI; (U) Antigen; (U) DNA; (U) Plasmid; (U) Hybridoma; (U) Toxin; (U) Immunoglobulin

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objective is to conduct studies on biochemical, molecular and cellular processes related to bacterial diseases of importance to the military. Molecules which may be by-products of the disease state, of the invading organism and of the immune system of the host are being identified and characterized. Types of molecules of interest include toxins, antigens, nucleic acids, enzymes, immunoglobulins; diagnostic tests and prophylaxis for military diseases are the envisioned products of this work.

24. (U) The approach includes the disciplines of biochemistry, microbiology, immunology and cell biology. Macromolecules are purified and characterized. Studies of virulence potential will be performed using cell-free enzyme assays, immunochemical assays and cell culture and animal toxicity assays, hybridoma technology and recombinant DNA technology is being applied to study of virulence factors.

25. (U) 83 10 - 84 09 Shiga toxin has been shown to inhibit elongation factor 1 dependent reactions of protein synthesis. The importance of KI capsule of E. coli in adult invasive disease has been established. The chemical structure of E. coli O25 antigen has been defined. Through application of molecular taxonomic technologies, several new species of Vibrio and Enterobacter have been established. Monoclonal antibodies to shiga toxin have been prepared and shown to react with toxins produced by E. coli causing hemorrhagic colitis and may be a useful basis for development of a useful diagnostic test for EPEC, hemorrhagic colitis and other E. coli producing shiga-like toxins. Plasmids controlling S. dysenteriae LPS antigen has been identified and characterized. Monoclonal antibodies to C. difficile A + B toxin have been prepared. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83 - 30 Sept 84.

PROJECT: 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

WORK UNIT: 210 Biochemical Research on Military Diseases

INVESTIGATORS:

Principal:	Peter Gemski, Ph.D.
Associates:	CPT James E. Brown, Ph.D., MSC; George R. Fanning, M.S.; SP5 Douglas A. Foret; John Kintzios, B.S.; Susan Kotarski, Ph.D.; SP6 William Mayo; SP4 Kate Kraus, B.S.; SP5 Timmie Merriwether; Roger J. Neill, Ph.D.; Sara Rothman, Ph.D.; Norma Serano, B.S.; Mary A. Sodd, M.S.; SP4 Dana Wells, B.S.
In collaboration with:	C. R. Alving, COL, M.C. (Dept. Membrane Bio-Chemistry); D.J. Brenner, Ph.D. (CDC, Atlanta, GA); A.S. Cross COL, M.C.,(CD&I); Mary K. Gentry, B.S.; SP4 Kara K. Kopec; Walter C. Hill, Ph.D. (FDA), Washington, D.C.; A. Lindberg, (Karolinska Institute, Stockholm, Sweden); T. G. Obrig, Ph.D. (Albany Medical College, Albany, NY).

DESCRIPTION:

The mission is to design and execute research programs that provide fundamental biochemical and molecular definitions of diseases relevant to the military. Factors associated with enteric disease processes such as virulence determinants and toxins of organisms, biochemical and metabolic mechanisms of enteric pathogens, and products of host responses to disease are being studied through the use of physiochemical, biochemical, microbiological and immunological concepts and techniques. Such information provides a rational basis for immunological and chemotherapeutic protection against disease and the development of accurate diagnostic procedures.

A. Studies of Shigella and Their Toxins.

1. Inhibition by Shiga Toxin of Elongation Factor 1-Dependent Reactions of Protein Synthesis.

Shiga toxin, a protein toxin of *Shigella dysenteriae* 1, previously shown to inhibit enzymatically eukaryotic protein synthesis, has now been examined for a direct effect on elongation factor 1(EF-1)-dependent reactions. Purified toxin from *S. dysenteriae* 1 strain 3818-0 was activated using TPCK-trypsin, followed by phenylmethylsulfonyl fluoride, 10 M urea and 10 mM dithiothreitol. Shiga toxin inhibited [^{3}H]-Phe-tRNA binding to ribosomes when tested in reaction mixtures containing poly(U), purified EF-1, and 0.5 M KCl-washed reticulocyte ribosomes. Binding was inhibited 24% and 68% by 0.045 and 0.45 μg toxin/ml, respectively. Similar levels of inhibition were observed with α-sarcin, whereas no inhibition was detected with diphtheria toxin. Inhibition by Shiga toxin was not affected by either rate-limiting or saturating amounts of EF-1 protein. Concentrations of toxin which inhibited EF-1-dependent Phe-tRNA binding caused similar reductions of total protein synthesis in reticulocyte lysate reaction mixtures. Non-enzymatic binding of Phe-tRNA to ribosomes was not affected. EF-1-dependent enzymatic GTPase was inhibited by Shiga toxin in a dose-dependent manner in mixtures containing EF-1, Phe-tRNA, ribosomes and [γ-^{32}] GTP. The degree of inhibition was equal to that observed with phytolaccin (PAP) or α-sarcin. We conclude that Shiga toxin has a direct inhibitory effect on aminoacyl-tRNA binding reactions of peptide elongation.

2. Reaction of Monoclonal Antibodies against Shiga Toxin with Cytotoxic Extracts of Escherichia coli and Shigella dysenteriae Strains.

Some *Escherichia coli* serotypes have been shown to produce a Shiga-like cytotoxin. We have analyzed cytotoxic extracts from two of these strains and from *Shigella dysenteriae* using monoclonal antibodies and monospecific antibody prepared against purified Shiga toxin. *E. coli* 0157: H7 strain 933 (hemorrhagic colitis), 0103:H2 strain S/22/1 (infant diarrhea), and *S. dysenteriae* strain 3818-0 were grown in deferrated medium and extracts were prepared by sonication. Cytotoxic activity was quantitated using the HeLa cell assay developed for Shiga toxin. Monoclonal antibodies to Shiga toxin neutralized cytotoxic extracts from these three strains. Immunochemical analysis using a western blot technique showed that each of the extracts from *E. coli* and *S. dysenteriae* contained a protein with the same electrophoretic mobility as pure Shiga toxin and with shared immunological determinants. These monoclonal antibodies should prove useful, not only for characterizing cytotoxins of *E. coli*, but also as probes for detecting the virulence factor in *E. coli* associated with disease.

3. Plasmids Encoding LPS Synthesis of Shigellae.

Progress has been made in constructing and identifying plasmids which contain genes encoding functions required for lipopolysaccharide (LPS) synthesis in *S. flexneri* and *S. dysenteriae*. Isolation of genetic information allowing a bacteria to mimic the LPS of *S. flexneri* and *S. dysenteriae* is of prime importance in constructing a live oral vaccine against these diarrhea causing bacteria. The chromosomal genes involved in synthesis of the group 3,4-0 antigen of *S. flexneri* were inserted by reciprocal recombination onto a F' plasmid originally derived from *E. coli*. The hybrid plasmid was transferred by conjugation at low frequency into recipient bacteria. Introduction of the hybrid plasmid into *E. coli* K12 and *S. typhimurium* resulted in expression of the group 3,4 antigen in these strains. In addition, from an *S. dysenteriae* strain we isolated a naturally occuring 25 kilobase plasmid which encoded resistance to streptomycin and a function required for Type 1 0-antigen synthesis in *S. dysenteriae*. This plasmid, designated pDOT1, was introduced into *E. coli* K12 strains by transformation or by conjugat-ion with a mobilizing plasmid. *E. coli* strains harboring pDOT1 produced some but not all of the immunological characteristics of Type 1 0-antigen of *S. dysenteriae* and exhibited a modified lipid A moiety. These preliminary genetic studies indicate that plasmids encoding functions involved in LPS synthesis can be transferred into and expressed in bacteria that may prove to be useful vaccine candidates.

B. Nucleotide Sequence Relatedness Among Enterobacteriaceae.

1. *Vibrio furnissi* Formerly Aerogenic Biogroup of *Vibrio fluvialis*, a new Species Isolated from Human Feces and the Environment.

Strains formerly classified as the aerogenic (gas-producing) biogroup of *Vibrio fluvialis* were shown by DNA relatedness to be a separate species. The species was named *Vibrio furnissi* sp. nov. (type strain ATCC 35016 = CDC B3215). Three strains of *V. furnissii* were 79% or more related to the type strain of *V. furnissii* and about 50% related to the type strain of *V. fluvialis* strains. *V. fluvialis* were 40 to 64% related to the type strain of *V. furnissii*. Divergence in related sequences was only 0.0 to 1.5% among strains of *V. furnissii* and among strains of *V. fluvialis* but was 5.0 to 8.0% in interspecific reactions between *V. fluvialis* and *V. furnissii*. *V. furnissii* was aerogenic (produced gas from the fermentation of carbo-hydrates). Another test of some help in differentiating the two species was fermentation of L-rhamnose (57% positive for *V. furnissii* and negative for *V. fluvialis*). In addition to the reactions above, *V. furnissii* is distinguished from other salt-requiring vibrios on the basis of its positive reactions in tests for Møller L-arginine, L-arabinose, maltose, and D-mannitol and its negative reactions for Møller L-lysine and L-ornithine, lactose, and Voges-Proskauer. *V. furnissii* has been isolated from patients with acute gastroenteritis in at least two outbreaks of food poisoning; its role as a cause of diarrhea needs further study.

2. Attempts to Classify Herbicola Group-*Enterobacter agglomerans* Strains by Deoxyribonucleic Acid Hybridization and Phenotypic Tests.

There are seven names on the Approved Lists of Bacterial Names that have been treated as partial or total synonyms for strains belonging to the *Enterobacter agglomeran*-Herbicola group of *Erwinia* species complex. A total of 124 strains belonging to this complex, isolated mainly from plant and human sources, were studied by deoxyribonucleic acid relatedness and by a variety of biochemical tests. Ninety of these strains fell into 13 deoxyribonucleic acid hybridization groups (2 to 13 strains per group), and the remaining 34 strains did not fit into any hybridization group. Nine of the hybridization groups could be separated biochemically, whereas four hybridization groups could not. Our results point out the inadequacy of the classification schemes presently used for these organisms, the inadequacy of the present nomenclature, the extreme diversity of the strains presently classified in the *Enterobacter agglomerans*-Herbicola group of *Erwinia* species complex, and the need for additional, in-depth studies of these organisms.

3. *Enterobacter taylorae*: A New Species of Enterobacteriaceae Isolated from Clinical Specimens.

The name *Enterobacter taylorae* (formerly known as Enteric Group 19) is proposed for a group of organisms isolated from various clinical sources as well as animals and the environment. By DNA hybridization, *E. taylorae* strains are 84-93% related to the type strain. Their nearest relative was *E. cloacae* which was 61% related. *E. taylorae* is distinguished from *E. aerogenes* and *E. cloacae* by its negative lysine decarboxylase, negative sucrose, sorbitol, raffinose, and melibiose fermentation reactions, and by its positive arginine dihydrolase. Biochemically atypical strains of *E. taylorae* were only 50-58% related to the type strain and will form the basis for three additional biogroups of *E. taylorae*. Preliminary antimicrobial susceptibility data shows the organism to be susceptible to most drugs with the exception of penicillin and cephalothin.

4. Enteric Group 45: A New Species of Enterobacteriaceae.

Enteric group 45 consists of 12 strains that fit the definition of *Enterobacteriaceae* and that were originally identified a "atypical *Hafnia alvei*." They were tested for DNA relatedness by the hydroxyapatite method using ^{32}P-labeled DNA. The 12 strains were 90% or more related in 60°C reactions. The divergence (unpaired bases) in related nucleotide sequences was 0.0-1.0%. Relatedness of Enteric group 45 to DNA hybridization reference strains of representative species of *Enterobacteriaceae*, including *H. alvei* (15%), was less than 35%. They differed from *Hafnia* in their resistance to Guinee's *Hafnia*-specific phage, positive methyl red reaction (22°C), positive Simmons' citrate and cellobiose reactions within 24 h, and negative Voges-Proskauer and Jordan's tartrate reactions. All strains were susceptible to naladixic acid, sulfadiazine, gentamycin, kanamycin, tetracycline, chloramphenicol, and carbenicillin (33% showed intermediate zones to streptomycin), but strains showed resistance to penicillin (100%), colistin (92%), cephalothin (83%), and ampicillin (42%). Six strains were isolated from arm or leg wounds, 3 from sputums, and one each from a stool, water, and an unknown source. Enteric group 45 will be proposed as a new species in the family *Enterobacteriaceae*.

5. Genetic Methods for the Detection of Microbial Pathogens: A Collaborative Study to Identify Enterotoxigenic *Escherichia coli* by DNA Colony Hybridizaton.

One determinant of virulence that enteropathogenic *Escherichia coli* may manifest is a heat-labile, cholera-like toxin (LT). The gene that codes for LT can be purified by recombinant DNA techniques and used as a genetic probe for DNA hybridization to detect strains that may not manifest toxin production but carry the genetic information to do so.

Thirteen laboratories received 3 known and 25 unknown (10 positive and 15 negative) cultures of *E. coli* to be tested for the presence or absence of the LT gene. These isolates had been tested and classified by

AVAILABLE

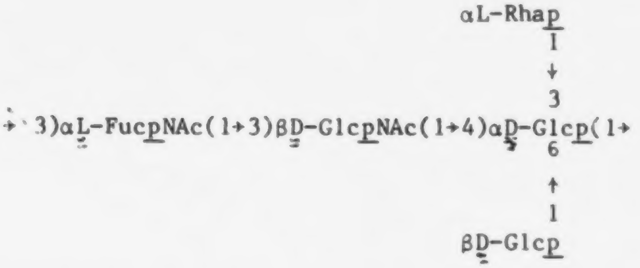
2 independent methods. Cultures were spotted on nitrocellulose filters on MacConkey agar and incubated. Colonies were lysed *in situ* and their DNA hybridized to ^{32}P-labeled, purified LT gene DNA (provided to the collaborators). Positive colonies were identified by autoradiography.

Of 325 samples, 315 (96.9%) were identified correctly. Ten samples were misclassified: 4 false negative and 6 false positive results. Chi-square values indicate that the method agrees with the previous classification and is equally efficient at classifying positive and negative samples (95.7% and 98.1%, respectively).

C. Studies of LPS and other Surface Antigens.

1. Structural Studies of the Escherichia coli O-Antigen 25.

The structure of the *Escherichia Coli* O-antigen 25 has been investigated using N.M.R. spectroscopy, methylation analysis and different specific degradations. It is concluded that the O-antigen is composed of pentasaccharide repeating-units having the structure:



$$\rightarrow 3)\alpha\text{L-Fuc}p\text{NAc}(1\rightarrow 3)\beta\text{D-Glc}p\text{NAc}(1\rightarrow 4)\alpha\text{D-}\underset{\substack{6\\ \uparrow\\ 1\\ \beta\text{D-Glc}p}}{\overset{\substack{\alpha\text{L-Rha}p\\ 1\\ \downarrow\\ 3}}{\text{Glc}p}}(1\rightarrow$$

2. The Importance of the K1 Capsule in Invasive Infections Caused by Escherichia coli.

We examined 534 clinical isolates of *Escherichia coli* for sensitivity to rough lipopolysaccharide-specific and K1-specific phages. Twenty-eight percent of bacteremic isolates were sensitive to rough-specific phages. Forty-two percent of these strains, against only 20% of bacteremic isolates insensitive to rough-specific phages, had K1 capsule ($P<0.001$). K1-positive strains were usually resistant to phagocytic killing, whereas strains lacking the K1 capsule were more likely to be killed regardless of capsular type. Eighty-two percent of strains were typable with O-specific, 57% with K-specific, and 74% with H-specific antisera. Sixty percent of *E. coli* were agglutinated by only 10 O-specific antisera. K1 was the most common capsular type, followed by K5, K2, and K12, whereas four H antigens accounted for nearly half of the H-typable strains. We conclude that (1) the combination of rough-specific and K1-specific phage sensitivity defines functionally similar groups of bacteria and (2) a polyvalent vaccine against invasive *E. coli* is possible given the relatively limited number of invasive O:K:H serotypes.

3. Importance of K1 Capsule in Serum Resistance of E. coli 012.

The ability of *E. coli* (EC) to resist killing by normal human serum (NHS) has traditionally been correlated with length of 0 side chain. In previous work (FEMS Microbiol. Letts. 9:193, 1980) we established an association of K1 capsule with rough LPS phenotype and hypothesized that the K1 capsule can confer to otherwise serum sensitive EC the ability to resist bactericidal kill. Of 234 bacteremic EC screened, we found 5 strains of the 012 serogroup. Each isolate was associated with a K1 capsule which led us to postulate that each had an altered LPS; however, all isolates were resistant to rough-specific phages and had 0 side chain on SDS-PAGE, indicating the presence of fully capped LPS cores (i.e. smooth LPS). Unlike the parental strains, 25 isogenic mutants lacking the K1 capsule were killed by NHS in the absence of neutrophils. In contrast, selection of 8 unencapsulated mutants from 4 different EC of the 06 serogroup that were K5 encapsulated retained their serum resistance. We conclude that (1) some EC 0 serogroups require encapsulation for serum resistance whereas other serogroups do not, and (2) LPS size alone is not always an important determinant of serum resistance. These data provide additional support to the hypothesis that K1 capsule protects otherwise serum sensitive EC from the bactericidal effect of NHS.

4. Identification of Escherichia coli K1 Antigen.

We compared the use of bacteriophage sensitivity, seroagglutination with polyclonal antisera raised in rabbits or horses, seroaggluntination with murine monoclonal antibody, and the serum agar precipitin technique for the detection of K1 capsular polysaccharide among clinical isoltes of *Escherichia coli* obtained from blood stream infections. Some *E. coli* isolates failed to yield agreement among these tests, indicating that reliable detection of K1 antigen may require the use of multiple tests.

5. A Possible Role For Anti-Capsular and Anti-Lipopolysaccharide Antibodies in Infection Caused by K1-Positive E. Coli.

We have previously shown that K1-encapsulated *E. coli* (K1EC) grown to late log phase are killed by neutrophils (PMN) in the presence of an IgM anti-K1 capsular monoclonal antibody but not with intravenous immune globulin (IVIG), normal human (NHS) or psot-K1 polysaccharide (PS) vaccinated or post-K1EC infection sera. (J. Inf. Dis. 147:68, 1983). In an effort to define conditions under which K1EC might be killed by PMN with NHS, we examined the role of bacterial number and growth phase in the killing of K1EC by PMN and NHS. Although K1 PS is detectable in early log phage growth, NHS and PMN were found to kill K1EC (mean kill 57 ± 29%, n=8). Bacterial killing was affected by PMN: bacterial ratio and was completely inhibited by homologous lipopolyasaccharide (LPS), but not by K1 PS. In contrast, the killing of late log phase K1EC by monoclonal antibody was inhibited by K1 PS but not by homologous LPS. Since we found antibody to the LOPS of major EC serotypes but not to K1 PS in 21 different commercial preparations of IVIG, IgG antibody to the PLS of invasive strains of EC appears to be present in the general population. We speculate that in early stages of infection when there may be small numbers of KlEC or early bacterial growth phase, the low level of natural anti-LPS antibody may be sufficient for PMN to kill KlEC; however, once infection with KlEC is established in the host, anti-Kl capsular antibody may be more effective than anti-LPS antibody for the killing of the organism by the host defenses. In this situation IVIG would not be expected to be efficacious and a source of anti-Kl capsular antibody, such as a monoclonal antibody preparation, might be considered.

6. Heterogeneity of Lipid A: Comparison of Lipis A Types from Different Gram-Negative bacteria.

Chloroform-soluble purified lipid A preparations from 10 sources, including five *Escherichia coli* strains (EH 100, K-12, 0127, 0111, RCDC), two *Salmonella* strains (*Salmonella typhimurium*, *Salmonella minnesota* R595), *Shigella sonnei* II, and a hybrid of *Shigella flexneri* and *E. coli* K-12, were compared with a lipid A from *S. flexneri*. Purified lipid A from *S. flexneri* was earlier found to be composed of eight fractions. The vrious lipid A preparations were assayed by thin-layer chromatography. Chromatograms were stained for phosphate or carbohydrate by molybdenum blue or orcinol, respectively. The number of major bands found for each lipid A Preparation varied between 7 and 10, depending on the source. Comparable bands, based on R_f, were found among all of the different lipid A preparations, but the quantity of each band varied between the sources of lipid A. Four bands (designated 2, 3, 7, and 8) were abundant in every preparation. Variations of conditions used for preparing lipid A, such as changing of hydrolysis time, did not affect the appearance of lipid A on thin-layer chromatography. Change in the type of acid used for hydrolysis also did not affect the band pattern, but it did change the quantitative amounts of the various bands to some degree.

Heterogeneity of Lipid A.

In a previous study, lipid A from *Shigella flexneri* was separated into eight distinct fractions by thin-layer chromatography (TLC). In the present investigation, lipid A from different gram-negative bacteria was analyzed by thin-layer chromatography for its heterogeneity and compared with lipid A of *S. flexneri*. Purified lipid A preparations from six different enterobacterial species were heterogeneous, but based on Rf, comparable bands were found among all preparations. Changing the culture conditions by using two other culture media resulted in very minor changes in the TLC appearance. Different serotypes of *Shigella* grown under the same conditions also showed very minor differences. Two bands (5 and 8) in each preparation of lipid A comigrated with lysophosphatidylethanolamine, and phosphatidylethanolamine, respectively, and both bands were stained with ninhydrin and molybdenum blue. Therefore, phosphatidylethanolamine, and probably lysophosphatidylethanolamine, are common constitutents of unfractioned lipid A preparations, and both phospholipids can occur even in highly purified unfractionated lipid A.

D. Studies of Clostridium difficile toxins.

The isolation and purification in our laboratories of large quantities of *Clostridium difficile* toxin A and toxin B gave us the reagents needed to produce monoclonal antibodies against these toxins. As both toxins are lethal for mice, they were toxoided with glutaraldehyde for priming. The resultant fusion resulted in production of over 120 cell lines producing antibody as measured by radioimmunoassay. All the antibodies are IgM.

Future Plans of the Department of Biological Chemistry (WWH2).

Continue studies of toxins, virulence factors, plasmids, monoclonal antibodies and antigens related to pathogens with emphasis on enteric diseases; continue genetic and cloning studies of virulence factors and antigens of pathogens for use in vaccines, gene probes and other products; continue to identify and characterize receptors for toxins.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OC 6744
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636
3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10 NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
PRIMARY	61102A	3M161102BS10	AH	211 WWH3
CONTRIBUTING				
~~CONTRIBUTING~~	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code): (U) Biochemistry of Parasitic Drugs
12. SUBJECT AREAS: 0601 Biochemistry 0613 Microbiology
13. START DATE: 78 10
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House
17. CONTRACT/GRANT
DATE EFFECTIVE:
EXPIRATION:
CONTRACT GRANT NUMBER:
TYPE:
d. AMOUNT:
KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	3.0	389
85	3.0	221

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Division of Biochemistry
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g. NAME OF ASSOCIATE INVESTIGATOR (if available): Jett, M

21. GENERAL USE: FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Dogs; (U) Rodents; (U) Antibody; (U) Parasites (U) Drug Carriers; (U) Liposomes; (U) Antiradiation Drugs; (U) Lab Animals; (U) RAMI;

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objective is to investigate biochemical aspects that influence lipids and membranes of parasitic diseases. The goal is to develop drug carriers that will deliver conventional antimicrobial drugs or serve as vehicles for vaccines. The main emphasis is to examine liposomes as drug carriers or as carriers of adjuvants and proteins for immunization. Adjuvants to be examined include lipid A from endotoxin and other lipidic or lipid-conjugated materials. The role of the arachidonic acid cascade in relation to the infectious process is to be examined, as well as how these compounds may influence the effectiveness of drug delivery. There is considerable military relevance in this research because liposomes are projected for use as drug carriers for treatment of leishmaniasis, and virus diseases (Rift Valley Fever), and possibly for delivery of protein antigens in a malaria vaccine.

24. (U) An attempt will be made to utilize liposomes as carriers of prostaglandins and leukotrienes and to examine the effects of liposome delivery on phospholipid metabolism in macrophages. Incorporation of protein antigens (malaria sporozoite antigen, and peptides of acetylcholinesterase) into liposomes and investigation of the immune response generated in animals will be performed.

25. (U) 83 10 - 84 09 Effects of parasite virulence on efficacy of liposome-encapsulated antimonial drug was examined in experimental leishmaniasis in hamsters. A novel method for assaying endotoxic lipids on thin layer chromatography plates utilizing enzyme-linked immunosorbent assay (ELISA) was developed. The influence of liposomal lipids on phospholipid metabolism of macrophages was examined. Decreased toxicity of liposome-encapsulated ribaviran in monkeys and increased efficacy against Rift Valley Fever virus infection in mice, was demonstrated. For technical report see WRAIR Annual Progress Report 1 Oct 83- 30 Sept 84.

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AVAILABLE

PROJECT: 3M161102BS10 BASIC RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS
WORK UNIT: 211 Biochemistry of Parasitic Drugs

INVESTIGATORS:
Principal: Carl R. Alving, M.D., COL, MC
Associates: Roberta L. Richards (Owens), Ph.D., DAC; Nabila M. Wassef, Ph.D., DAC; Marti Jett, Ph.D., DAC.
Assistants: SP4 Pearl Burke

DESCRIPTION:

The major goal was to develop liposomes as drug carriers in treatment of parasitic diseases especially leishmaniasis. The influence of liposomes on the immune system was also examined because of our discovery that antibodies against liposomes could be generated by lipid A (endotoxin). The possible feasibility of using liposomes containing lipid A as vehicles for vaccines was examined.

1. Liposomes in leishmaniasis: effects of parasite virulence on treatment of experimental leishmaniasis in hamsters

During studies on the use of liposomes as drug carriers in experimental leishmaniasis in hamsters, we noted incidentally that the apparent virulence of the infection often varied widely between different large groups of animals. When the death rates among control animals (injected only with saline) were compared with hepatic parasite counts of survivors in the same group, three distinctive types of infection were observed: type I, low death rate, low parasite count in survivors; type II, high death rate, low parasite count in survivors; type III, high death rate, high parasite count in survivors. The apparent virulence, based on death rates both at early and late stages of infection, was in the order I<II<III. Therapeutic efficacy of a drug (meglumine antimoniate) or liposome-encapsulated drug against each type of infection was in the order I>II>III. Liposomes reduced the drug dose required for each infection type many hundred-fold and reduced the death rate for type I to zero. However, among animals with type III (or even type II) infection certain individuals were completely refractory to treatment, even when liposome-encapsulated drug was employed, and the lowest mortality rate achieved was approximately 30%. This latter resistance to treatment may have been due to irreversible tissue damage caused by advanced disease, or it may have reflected resistance of certain virulent infections to treatment.

2. Antileishmanial activity of liposome-encapsulated meglumine antimonate in the dog

Experimental infections of Leishmania donovani in mixed-breed dogs were induced to determine the antileishmanial efficacy of liposome-encapsulated meglumine antimoniate (LEMA). Each dog was inoculated IV with $1.0 \pm 0.2 \times 10^8$ amastigotes of a Khartoum strain of L. donovani/kg of body weight. The antileishmanial agents (LEMA or unencapsulated meglumine antimoniate) were given once daily, IV, for 1, 4, or 10 consecutive days beginning the 12th day after inoculation. The dogs were killed 3 or 4 days after completion of therapy, and parasites in the spleens were quantified.

A single injection of LEMA (0.61 mg of Sb/kg of body weight) resulted in 89% suppression and 4 consecutive daily injections of LEMA (1.94 mg of Sb/kg/day) resulted in 95.8% suppression of splenic parasites. The dose of LEMA that would give 50% suppression (SD_{50}) was estimated as approximately 0.029 mg of Sb/kg. The SD_{50} for unencapsulated drug was estimated as approximately 24 mg of Sb/kg. The liposome-encapsulated drug was estimated to be more than 700 times more efficacious than the unencapsulated drug. Seemingly, liposomes can markedly reduce the drug dosage required for equivalent.

3. Natural antibodies against phospholipids and liposomes in humans

The data provide evidence that naturally occurring antibodies against some of the most fundamental membrane phospholipids are widespread in normal human sera. The data also support the concept that naturally occurring anti-liposome antibodies represent a variety of specificities against different phospholipids, but that factors such as membrane charge and membrane fluidity modulate the binding of the antibodies to particular lipid membranes. The natural antibodies described here thus are similar to the monoclonal anti-liposome antibodies experimentally induced by injecting liposomes containing lipid A. All of these findings support our previous hypothesis that antibodies against lipids may represent a novel group of autoantibodies that have important physiological and biological functions.

4. Lipid A fractions analyzed by a technique involving thin-layer chromatography and enzyme-linked immunosorbent assay

A modified enzyme-linked immunosorbent assay (ELISA), with alkaline phosphatase as enzyme, was used for the study of antigenicity of lipid A fractions directly on thin-layer chromatographic (TLC) plates. For visualization a gel slab containing the enzyme substrate was placed on the plate containing enzyme-conjugated antibodies. The plate was read by a thin-layer chromatogram spectrophotometer. The immunoassay was both highly specific and quite sensitive. Sensitivity was superior to levels obtained by staining the plate with molybdenum blue (for phosphate) or orcinol (for carbohydrate). Fractions of lipid A from *Escherichia coli* O111, *Shigella flexneri* or *Salmonella minnesota* R595, after being separated by thin-layer chromatography, were analyzed using rabbit anti-(lipid A) serum. Patterns obtained by scanning the same plates for phosphate staining and for the TLC-ELISA corresponded well. For comparison with TLC-ELISA, an inhibition assay was run using a tube ELISA. The tube ELISA, run in aqueous medium, showed that fractions 6 - 8 (those having the highest R_F values) had the least activites. In contrast, TLC-ELISA did not detect large differences between fractions 2 - 7. This discrepancy probably reflected limited aqueous solubility of fractions 6 and 7. We conclude that TLC-ELISA might reveal antigenic activities of lipids that could be missed by other methods. The data suggested that all fractions, except for fraction 8, were similar in their antigenicity by TLC-ELISA. Differences in antigenicity between the fractions occurred when the fractions were tested in free form in an aqueous environment and these differences possibly could have been due to different solubilities of individual fractions.

5. Heterogeneity of Lipid A: Comparison of Lipid A Types from Different Gram-Negative Bacteria

Chloroform-soluble purified lipid A preparations from 10 sources, including five *Escherichia coli* strains (EH100, K-12, 0127, 0111, RCDC), two *Salmonella* strains (*Salmonella typhimurium*, *Salmonella minnesota* R595), *Shigella sonnei* II, and a hybrid of *Shigella flexneri* and *E. coli* K-12, were compared with lipid A from *S. flexneri*. Purified lipid A from *S. flexneri* was earlier found to be composed of eight fractions. The various lipid A preparations were assayed by thin-layer chromatography. Chromatograms were stained for phosphate or carbohydrate by molybdenus blue or orcinol, respectively. The number of major bands found for each lipid A preparation varied between 7 and 10, depending on the source. Comparable bands, based on R_F, were found among all of the different lipid A preparations, but the quantity of each band varied between the sources of lipid A. Four bands (designated 2, 3, 7, and 8) were abundant in every preparation. Variations of conditions used for preparing lipid A, such as changing of hydrolysis time, did not affect the appearance of lipid A on thin-layer chromatography. Change in the type of acid used for hydrolysis also did not affect the band pattern, but it did change the quantitative amounts of the various bands to some degree.

6. Heterogeneity of Lipid A

In a previous study, lipid A from *Shigella flexneri* was separated into eight distinct fractions by thin-layer chromatography (TLC). In the present investigation, lipid A from different gram-negative bacteria was analyzed by thin-layer chromatography for its heterogeneity and compared with lipid A of *S. flexneri*. Purified lipid A preparations from six different enterobacterial species were heterogeneous, but based on Rf, comparable bands were found among all preparations. Changing the culture conditions by using two other culture media resulted in very minor changes in the TLC appearance. Different serotyopes of *Shigella* grown under the same conditions also showed very minor differences. Two bands (5 and 8) in each preparation of lipid A comigrated with lysophosphatidylethanolamine and phosphatidylethanolamine, respectively, and both bands were stained with ninhydrin and molybdenum blue. Therefore, phosphatidylethanolamine, and probably lysophosphatidylethanolamine, are common constitutents of unfractioned lipid A preparations, and both phospholipids can occur even in highly purified unfractionated lipid A.

7. Enhancement by Lipid A of Mucosal Immunogenicity of Liposome-Associated Cholera Toxin

Two methods that might enhance the mucosal immunogenicity of a protein antigen, cholera toxin (CT), were studied in rats: association of CT with liposomes, and coadministration of CT with lipid A. Enteric priming by CT was not enhanced when the antigen was trapped within liposomes or bound to their surface via G_{M1} ganglioside, nor was it improved when CT was mixed with lipid A or with liposomes containing lipid A. However, lipid A did enhance priming by liposome-associated CT when the lipid A was incorporated into CT-bearing liposomes. It is concluded that lipid A can act as an adjuvant for a local IgA response to a mucosally applied antigen, at least when lipid A and the antigen are associated on a liposome carrier.

8. Antibodies to Lipid A: Occurrence in Humans

Lipid A, the toxic part of the bacterial endotoxin, is a common antigen for many gram-negative bacteria. Antibodies to lipid A occur naturally in humans; they have been found in 10% - 34%, and even up to 73%, of individuals tested, as detected by indirect hemolysis and enzyme-linked immunosorbent assay (ELISA), respectively. Inflammatory bowel diseases (Crohn's disease or ulcerative colitis) cause changes in the level of antibodies to lipid A, as compared with that found in healthy control subjects. Increased levels of antibodies to lipid A are seen in both children and adults with infections due to gram-negative bacteria, such as urinary tract infections (UTI). The highest titers of IgG in serum, as detected by ELISA, have been recorded in patients with development or progression of renal scarring associated with UTI. Since lipid A may play a role in the pathogenesis of renal impairment, the determination of the level of antibodies to lipid A may help in the diagnosis of certain forms of UTI. Possible beneficial roles of antibodies to lipid A during septicemia caused by gram-negative bacteria in humans are still unclear.

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PATENT

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

				DA OC 6472	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
83 10 01	4. KIND OF SUMMARY D.Change	SUMMARY SCTY U	6. WORK SECURITY U	REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER	
	61102 A	3M161102BS10	CG	212	WWI6
CONTRIBUTING XXXXXXXXXXX	STOG 82/83-6.2/2				

(U)Physiology of Systemic Effects of Blast Overpressure

0621 Weapons Effects 0601 Biochemistry 0619 Stress Physiology

78 03	Cont	DA		C. In-house

	84	3.0	300
	85	3.0	307

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Mundie, T G

g. NAME OF ASSOCIATE INVESTIGATOR (if available)
Dodd, R T

(U) Blast Overpressure; (U) Pulmonary Biochemistry; (U) Pulmonary Receptors; (U) Impulse Noise; (U) Biophysical Modeling; (U) Lab animals; (U)RAM (U) Rabbits; (U) Cats; (U) Sheep; (U) Goats

23. (U) To define the physiologic effects of blast overpressure and to determine the limits of human safety for exposure to impulse noise. To develop a mathematical model of the thoraco-abdominal response to blast waves of military importance.

24. (U) Approach uses biochemical assays and physiologic tests before and after blast injury. Blood is analyzed for enzymes, elastin-related products and protein changes detected by 2 dimensional gel electrophoresis. Pulmonary tissue is examined histologically. A finite element model of the sheep and human torso will be developed. Engineering material properties of lung parenchyma are to be determined.

25. (U) 83 10 - 84 09, The possibility of a blood borne protein moiety marker of blast injury is being evaluated by screening animal serum samples with two-dimensional gel electrophoresis. A finite element model (FEM) of the sheep thorax has demonstrated an ability superior to that of the lumped parameter model in predicting intrathoracic pressures of sheep exposed to a variety of blast waves. The FEM has predicted the temporal and spatial alterations in intrathoracic pressure to be expected within lung parenchyma. Preliminary experiments using the water jet to impulsively load the sheep thorax and measuring transparenchymal pressures have qualitatively supported the FEM predictions. The visual display of model output has been improved with color graphics. In the study of blast effects inside defeated armored vehicles pressures were measured on the surface of an anthropomorphic shape and in the thoracic esophagus of sheep. These data will be used to assess the FEM's ability to handle complex pressure environments. In vitro modeling of gastrointestinal blast injury continues with refinement of the impact chamber; development of a viable isolated, perfused gut preparation; and embarkation on a protocol to study the correlates of injury. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

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PROJECT 3M161102BS10: RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 212: Physiology of Systemic Effects of Blast Overpressure

Principal Investigator: Yancy Y Phillips, M.D., MAJ(P), MC

Associate Investigators: Robert F. Hoyt, Jr., D.V.M., M.S., MAJ, VC
Thomas G. Mundie, Ph.D., CPT, MSC
Kenneth T. Dodd, Ph.D., GS-12

Problem Statement and Objectives

Certain Army weapon systems, some currently in use, others still in development, produce levels of blast overpressure which exceed the limits defined in MIL- STD-1474B "Noise Limits for Army Materiel". The research objective of the Department of Respiratory Research is to define the risk of non-auditory injury to crew members from the blast overpressure produced by these weapon systems. To obtain a general understanding of the interaction of blast waves with crew members, mathematical models are being developed using the inherent biomechanical properties of the human structure. Validation of the model's predictions will be made at frequent intervals to avoid costly development along non-productive paths. Biochemical indicators of non-auditory blast injury are also being sought in order to increase the probability of detecting subclinical damage and to reduce the complexity and invasiveness of current techniques for assessing injury.

Progress and Accomplishments in FY84

1. JAYCOR has continued their work on the EITAC computer code. This is a general purpose code for calculation of multi-dimensional, time-dependent, one- and two-phase flows. It is used to define pressure time histories for blast-shape interaction to include changes in shape orientation, comparison with actual field data and pressure wave propagation as a function of time.

2. The Finite Element Model (FEM) of the thorax has continued to be refined for improvements in transient predictions of timing and location of lung overpressures and of chest wall deformation. The graphics display of the FEM data has been

enhanced with color and increased resolution. A computer generated movie of the FEM predicted chest wall motion and lung overpressure histories has been produced. The FEM has also been used to predict the pressures developed on the pleural surface of the lung under ribs and associated intercostal muscle.

3. A symposium addressing the modeling of non-auditory blast injury was sponsored by the Department of Respiratory Research. Presentations were made by WRAIR and JAYCOR scientists.

4. Contractual research continues on development of a quantatitive evaluation of lung injury. Present thrust involves a gravimetric method to continuously measure total lung weight as an index of rate of progress of edema formation. In this manner the time-dependent consequences of various lung insults can be measured.

5. A study was conducted at the Lovelace Inhalation Toxicology Research Institute (ITRI) to elucidate biochemical markers of blast injury. Animals were exposed to single shots at various combinations of peak pressure and impulse. Blood samples were collected for analysis of angiotensin I converting enzyme and desmosine by radioimmunoassay and nonspecific proteins by 2-D gel electrophoresis.

6. A study has commenced to measure the lung parenchymal response to loading using a water jet impactor and freefield blast. The objectives of the protocol are to develop a technique for placing pressure sensors into the pulmonary airways and to compare the resulting trans-parenchymal and intrathoracic pressure signals from both simulated and freefield blast with those obtained from computer predicted intrathoracic pressure distribution for simulated "force" loading. These data will be used to validate the water jet impactor and the FEM.

7. Extramural work on the pathophysiology of blast induced gastrointestinal injury produced several major milestones in FY84. A viable isolated, perfused gut preparation was developed in the rabbit. This modeling coupled with a water chamber blast simulating impactor system has facilitated the real time _in vitro_ study of injury mechanisms.

Recommendations and Objectives for FY85

1. Field and laboratory studies will measure thoracic wall motion in impulse and blast loading simultaneous with measurements of trans-parenchymal pressures.

2. Analysis of candidate biochemical markers of blast injury will be completed.

3. The sheep thorax FEM will be assessed with actual experimental results in simple environments matching intrathoracic pressures and chest wall motion parameters.

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2. Phillips Y. Y: Nonauditory effects of repeated exposure to intense impulse noise. Presented at the Fifth Annual Meeting of RSG-6 (NATO Panel VIII), Meppen, FRG, May 1984.

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5. Hoyt, R.F., Jr. and Yonushonis, W.P.: Techniques for the chronic lung lymph preparation in sheep. Presented at 34th Annual Session of American Association for Laboratory Animal Science, San Antonio, TX, Nov 1983.

6. Hoyt, R.F., Jr.: Sheep lung lymph cannulation. Presented at 36th Annual Carl W. Tempel Symposium on Pulmonary Disease and Allergy Immunology, FAMC, Aurora, Colo, Jan 1984.

7. Hoyt, R.F., Jr.: Cricothyroidotomy, tracheotomy, and tracheostomy. Emergency War Surgery Training Program, Symposum on Military Veterinary Medicine, WRAIR, Washington, D. C., April 1984.

8. Hoyt, R.F., Jr.: Sheep lung lymph collection: preparation and uses. Presented at 13th Annual NCAB/AALAS Seminar, Hunt Valley, Maryland, Sept., 1984.

9. Hoyt, R.F., Jr.: Cryosurgery. Presented at 13th Annual NCAB/AALAS Seminar, Hunt Valley, Maryland, Sept. 1984.

PUBLISHED ABSTRACTS

1. Phillips, Y.Y: Investigating hypoxemia, shunt and low V/Q - a 3 compartment lung model. In Proceedings of 36th Annual Carl W. Tempel Symposium on Pulmonary Disease and Allergy Immunology, FAMC, Anrora Colo., Jan 1984.

2. Rosenthal, R.R., Laube, B.L., Jaeger, J.J., Phillips, Y.Y, and Norman, P.S.. "Methacholine sensitivity is unchanged during the refractory period following an exercise or isocapnic challenge." Am. Rev. Resp. Dis 129 (42) 250, 1984.

3. Young, A.J., Hoyt, R.F., Jr., Jaeger, J.J., and Richmond, D.: Pulmonary microvascular permeability following short duration airblast. Abstract presented at FASEB, April, 1984.

4. Hoyt, R.F., Jr. and Yonushonis, W.P.: Techniques for the chronic lung lymph preparation in sheep. Abstract of scientific papers 34th Annual Session of American Association for Laboratory Animal Science, San Antonio, TX, Nov. 1983.

5. Hoyt, R.F., Jr.: Sheep lung lymph cannulation. In Proceedings of 36th Annual Carl W. Tempel Symposium on Pulmonary Disease and Allergy Immunology, FAMC, Aurora, Colo, Jan, 1984.

6. Hoyt, R.F., Jr.: Young, A.J., Jaeger, J.J., and Phillips, Y.Y: Pulmonary microvascular permeability following low level airblast exposure. WRAIR Research Report, Vol 4(4):2, Jan, 1984.

7. Gross, D.R., Van Oort, G., and Dodd, K.T.: Large changes in distal aortic longitudional segment length associated with breathing in the dog. American Heart Assoc. 36th Scientific Session, Nov., 1983.

8. Gross, D.R., Van Oort, G., Dodd, K.T.: Effects of simulated exercise on the pressure diameter relationship of the terminal aorta in dogs American Heart Assoc. 36th Scientific Session, Nov, 1983.

9. Gross, D.R., Van Oort, G., Dodd, K.T.: Changes in distal aortic mechanical properties associated with handling position in the dog. American Heart Assoc. 36th Scientific Session, Nov, 1983.

10. Gross, D.R., Van Oort, G., Dodd, K.T.: Changes in lower limp movement as a potential source for terminal aorta artherogeneses. 1984 American Council of Sports Medicine Annual Meeting.

11. Gross, D.R., Dodd, K.T., Van Oort, G., P.R., Wella, D.W., Fife, W.P.: Hemodynamic effects of 10% dextose and dextrase 70 on hemorrhagic shock during exposure to hyperbaric air and hyperbaric hyperoxia, aviation, Space and Environmental Medicine (In Press).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OC 6451	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	CD	213 WWJA
b. CONTRIBUTING				
~~c. CONTRIBUTING~~	STOG 82/83-6.2/2			

11. TITLE (Precede with Security Classification Code)
(U) Biological Modulation of Military Performance

12. SUBJECT AREAS
0616 Physiology 0619 Stress Physiology 0510 Psychology

13. START DATE 76 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	7.0	718
85	7.0	921

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
Top, F H Jr
d. TELEPHONE NUMBER (include area code)
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
Division of Neuropsychiatry
b. ADDRESS
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
Elsmore, T F
d. TELEPHONE NUMBER (include area code)
(202)-576-2483
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
Hursh, S R
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
Wylie, R M

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U)Neuropsychiatry;(U)Physiology;(U)Performance;(U)Neurophysiology;(U)Neuroanatomy;(U)Stress;(U)Lab Animals;(U)Rats;(U)Monkeys;(U)RAM III

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Investigations will seek to describe the means by which the nervous system effects bodily responses to stress and injury, and to discern those combinations of physiologic and environmental parameters which collectively define the optimal conditions for effective military performance.

24. (U) Animal models of performance will be created using the techniques of operant and respondent conditioning and the role of internal factors in performance variability assessed by neurophysiologic recording of intracellular and extracellular bioelectric potentials; the descriptive and experimental neuroanatomical techniques of light and electron microscopy and histochemistry; stimulation or lesioning of discrete brain area; experimental modifications of nervous system and hormonal status by organ ablation and/or administration of exogenous hormones or other drugs; and techniques of chronobiology.

25. (U) 83 10 - 84 09 Major findings: A technique for rapid generation of demand curves for the analysis of economic variables in the control of behavior has been developed and validated in both rats and monkeys. Application of economic concepts to behavior in continuous environments such as those encountered in military operations appears to offer advantages to previous approaches to these problems. Analysis of brain mechanisms underlying memory has shown that parallel amagdylodiencephalic and hippocampo-diencephalic circuits are involved in the control of visual recognition memory. Ongoing studies on neural mechanisms underlying the control of limb movement have shown that monkeys can successfully track targets moving along a parabolic pathway which has i implications for the type of control system required to control limb movements. Initial studies with an open loop paradigm indicate that control is continuous, not sampled as has been sugested by some workers. For Technical report see Walter Reed Army Institute of Research Annual Report, 1 Oct 83 - 30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

Project: 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 213: Biological Modulation of Military Performance

Investigators:
Principal: Elsmore, T.F. Ph.D.
Associate: Hursh, MAJ, S.R.; Campbell, LTC, C.B.G.; Raslear, CPT, T.G.; Leu, CPT, J.R.; Petras, J.M., Ph.D.; Wylie, R.M., Ph.D.

Objectives:

The objectives of this project include the definition of the means by which the nervous system mediates bodily responses to stress and injury, and to discern those combinations of physiologic parameters which collectively define the optimal conditions for effective military performance. A major thrust of research in this work unit is the development of animal behavior models that more closely approximate realistic conditions outside of the laboratory. Techniques and methods are drawn from a broad spectrum of neuroscience disciplines including psychology, neurophysiology, neuroanatomy, neuropharmacology, and chronobiology.

Progress:

Adequate understanding of the biological bases of military performance requires a thorough understanding of neuroanatomy. We have been concerned this year with identification of neural systems underlying the rhythmic processes of respiration and circadian pacemaker systems. Coordinates for placing lesions or chemical tracer substances in the rat suprachiasmatic nuclei of the hypothalamus were determined and some preliminary data on projections of these nuclear groups obtained. Attempts to approach the pineal for similar purposes have been frustrating in the rat because the area is surrounded by large blood vessels. New efforts are underway on the use of fluorescent dyes for tract tracing. These are normally used for retrograde labeling of cell bodies of origin. We have found that bisbenzimide introduced into the globe of the eye appears to be transported anterogradely in the retinal ganglion cell axons and is transferred to accompanying glial cells and transneuronally to the target neurons of these axons. There is a suggestion that it may be transferred to the tertiary neuron in the system, as fluorescent neurons were found in what appears to be visual cortex. These findings are preliminary, but if correct, this technique would be a great improvement over current methods where lesions or chemical tracers must be introduced into each level of a multineuronal system to work out the whole pathway.

Studies of the brain mechanisms involved in spatial memory in monkeys are drawing to a close. These studies have shown that parallel amygdalodiencephalic and hippocampo-diencephalic circuits are involved in the control of visual recognition memory.

A continuing series of studies on the neural control of limb movement has focused this year on developing and assessing different models of the neural control systems involved. Computer simulations have been used to generate theoretical performance curves for the tracking performance of animals that are trained to point at targets moving along step, ramp, or parabolic trajectories. Comparisons of these theoretical curves with actual performance of monkeys has been inconclusive regarding the type of control system involved. Both a closed loop, negative feedback system or the same system operated with the feedback pathway open can produce reasonable approximations to the responses generated by the monkeys.

In our attempts to develop realistic animal models of military performance it has become necessary to consider continuous performance situations. Conventional theories of behavior appear to be inadequate for these situations, and consequently we have borrowed some concepts from economics and are in the process of evaluating them. Several studies of behavior have been initiated to directly compare open (conventional) and closed (continuous) experimental systems with subjects working for food. Data to date indicate that price increases produce monotonic response rate decreases in open systems and bitonic rate changes in closed systems. Several studies of the interactions among different commodities in choice situations are continuing. Substitution and complementary relations are found even when the commodities have no obvious physiological connection, such as eating and running in a running wheel for rats. This parallels common assertions from economic theory that have never before been verified in the laboratory. Initial studies on demand curves have been done showing how this approach may be used to characterize the motivational effects of a variety of physiological variables including stress, sleep deprivation, circadian rhythms and brain injury. In another study involving a continuous performance environment, it was shown that short-term and spatial memory in rats shows significant variations across the day.

Future objectives:

Studies on the neural bases of respiration, circadian rhythms, movement, and memory will continue. New fluorescent dye techniques will be compared with existing techniques for sensitivity and selectivity. These techniques will be applied to the tracing of neural tracts in both brain and spinal cord. Applicability and utility of economic concepts in the analysis of behavior will continue to be investigated, with an emphasis upon the use of these techniques in the development of realistic animal performance models.

Presentations

Bauman, R. A. (1984) The effect of availability of food on the alternation of lever presses. Eastern Psychological Association, Baltimore.

Elsmore, T. F. (1984) Animal models of human performance: structural and functional approaches to extrapolating from animal to man. Ninth Psychology in the DOD symposium, Colorado Springs.

Elsmore, T. F. (1984) Circadian rhythms in multiple fixed-ratio performance. Association for Behavior Analysis, Nashville.

Elsmore, T. F. (1984) Laboratory control and data acquisition with SKED on PDP8 and PDP11 computers. Association for Behavior Analysis, Nashville.

Hursh, S. R. (1984) Demand elasticity as a behavioral concept. Invited paper presented at the Experimental Analysis of Behavior Group Annual Conference, University of Sussex, Brighton, England.

Hursh, Steven R., Bauman, Richard A., Leu, John R., & Raslear, Thomas G. (1983). Demand curves for the analysis of reinforcement. Presented at the annual meeting of The Psychonomic Society.

Publications

Bachevalier, J., Parkinson, J. K., Aggleston, J. P., and Mishkin, M. Severe recognition impairment after combined but not separate transection of the fornix and the amygdalofugal pathways. *Experimental brain research,* in press.

Campbell, C. B. G. Parcellation theory: new wine in old wineskins. *Behavioral and brain sciences,* in press.

Campbell, C. B. G. Behaviorism and natural selection. *Behavioral and brain sciences,* in press.

Hamilton, B. E. and Natelson, B. H. (1984) Ultradian rhythms of gastric activity. *Pavlovian journal of biological science,* **10**, 32-35.

Hursh, S. R. (1983) Maximization and reinforcement theory compared. *Behavioral and brain sciences,* **6**, 324-326.

Hursh, S. R. (1984) Review of 'Behavioral regulation and learned performance: some misapprehensions and disagreements' by William Timberlake. *Journal of the experimental analysis of behavior,* **42**, 376-377.

Hursh, S. R. Behavioral economics. *Journal of the experimental analysis of behavior*, in press.

Hursh, S. R. and Bauman, R. A. The behavioral analysis of demand. In H. Rachlin and L. Green (Eds.), *Advances in behavioral economics, Vol i.*, Norwood NJ: Ablex Publishing Corp., in press.

Leu, J. R. (1984). Performance of rats in a radial arm maze varies with time of day. *Society for Neuroscience Abstracts*, **10**, 146.

Wylie, R. M. (1984) Visual tracking by monkeys: feedback control or open loop? *Society for Neuroscience Abstracts*, **10**, 341.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	DA OG 6755	2. DATE OF SUMMARY	84 10 01	REPORT CONTROL SYMBOL	DD-DR&E(AR) 636	
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	CE	214 WWJE
b. CONTRIBUTING				
c. XXXXXXXXXX	STOG 8[illegible]/83-6.2/2			

11. TITLE *(Precede with Security Classification Code)*
(U) Millimeter Wave Biophysics and Biohazards

12. SUBJECT AREAS
0618 Radiobiol 0616 Physiol 0705 Rad Chem

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	2.0	328
85	1.5	384

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)*
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER *(include area code)*
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
Dept of Microwave Research
b. ADDRESS
Div of Neuropsychiatry
Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
LARSEN, L E
d. TELEPHONE NUMBER *(include area code)*
(202)-576-3615

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION H

f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
RAFFERTY, C N
g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Biophysics; (U) Millimeter Wave; (U) Bioeffects; (U) Permittivity; (U) RAM III

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The objectives of the millimeter wave bioeffects program are to (1) establish a technology base in millimeter wave instrumentation as needed for biophysical research in this region of the electromagnetic spectrum, (2) to develop millimeter wave exposure systems for use with biological specimens under conditions of both continuous wave and high peak power operations, and (3) to explore biological hazards with special interest in the eye. The military relevance in this research derives from millimeter wave radar.

24. (U) The millimeter wave instrumentation system will consist of a millimeter wave phase locked synthesizer for the range 40-60 GHz. This will serve as the source for a six-port network analyzer that will provide network analysis based description of biological dielectrics in vitro. The continuous wave exposure system will consist of a 35 GHz, 1 kilowatt klystron amplifier, a 10 watt traveling wave tube driver and a 100 milliwatt Gunn diode oscillator. The pulse transmitter will consist of a 35 GHz traveling wave tube amplifier of 30 kilowatts peak power and 3 kilowatts average power. The antenna will consist of a WR 28 feed to an elliptical reflector. The biological hazard studies will emphasize two features: (1) The direct heating action of millimeter waves on the cornea of the eye and (2) the production of thermoacoustic expansion in cornea, lens and retina.

25. (U) 83 10 - 84 09 Development of the 35 GHz pulsed transmitters is complete. Preliminary millimeter wave (35 GHz) studies of ocular hazard indicate anterior chamber pathology at power densities ca 1 order of magnitude below those at 3 GHz. Spectral scanning equipment development has progressed into the system integration phase. The 40-60 GHz six-port network analyzer and digital synthesizer subsystems are completed, and have gone to system integration and modification for pulse mode operation. A biophysical chemistry lab and program have begun. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project 3M161102BS10 RESEARCH ON MILITARY DISEASE,
INJURY AND HEALTH HAZARDS

Work Unit 214: Millimeter Wave Biophysics and Biohazards

Investigators.
Principal: COL Lawrence E. Larsen, MD
Associate: Charles N. Rafferty, Ph.D.

Introduction

The millimeter wave program began as an unfunded requirement in 1979/1980. It was funded in FY 1981 at which time a MCA for new laboratory space was completed on time and on budget. The program consists of a biophysical segment which is still in the hardware development phase; and a biohazards segment which has only recently emerged from its hardware development phase. Also a new biophysical chemistry program has just completed the equipping of a laboratory located in Forest Glen.

Dielectric Relaxation

This program is designed to develop the first biosystem dielectric data base in the 40 to 60 GHz range. The hardware developments needed to support this goal consist of a 40 to 60 GHz digital synthesizer of previously unobtainable accuracy, a 40 to 60 GHz six port network analyzer, and a system integration step to include automatic process control as well as error correction. These steps have recently been completed; and an additional requirement has been added to accommodate dielectric measurements under transient conditions.

New physical theories have also been developed in the context of this program. This theoretical thrust has been directed to the issue of dielectric properties, under transient conditions. All existing theory is limited to steady-state conditions. The new transient theory depends upon cooperative effects at the molecular level. This effect produces energy deposition in the dielectric that is not accountable in normal Debye theory

Biophysical Chemistry

This program is designed to investigate conformational state changes in micromolecules consequent to exposure in dual beam systems which use RF power as either an effector or as a sensor in combination with optical beams. The laboratory needed to accomplish these objectives is located in space formerly occupied by neurochemistry at Forest Glen. The equipment needed for this laboratory has been acquired over the last two years. In vitro exposure systems have been developed at ca 35 GHz, 1 GHz, and ca 10 GHz. The first of these has completed also a dual beam chamber which combines optical and RF radiation as sensor and effector, respectively.

Millimeter Wave Biohazards

This program has developed the first high power pulsed millimeter wave exposure system for biomedical use in the free world. The program goal is to explore the limitations of safety standard based on continuous wave exposure and whole body averaged dosimetry. It consists of a 30 kilowatt pulsed transmitter, a 100 dB anechoic chamber tested from 18 to 100 GHz, a polarization twist reflector antenna, a staring infra-red radiometer, and closed circuit TV for subject monitoring. The frequency of operation is 35 GHz.

The site of greatest hazard to high power millimeter wave exposure is thought to be the cornea. The cornea of anesthetized rabbit subjects were examined in a pilot study.

Although the data is very preliminary, it does appear that pulsed millimeter wave exposures produce acute iridocyclitis at much lower power densities than similar studies in the same experimental animal with continuous wave exposures at 2450 MHz performed in this department about ten years ago. The millimeter wave exposure animals also demonstrated extensive indothelial epithelial histopathology which was not present with the 2450 MHz studies. These studies are presently in the stage of replication. However, progress is slow due to the staff shortages and equipment failure.

PUBLICATIONS

1. Larsen, L.E.; Guo, T.C.; Guo, W.W.; Kak, A.C., "Microwave Imagery Systems for Medical Diagnostic Applications", Special Publication of IEEE Transaction Biomedical Engineer, IEEE #84-CH-2058-6, pp 532-536, 1984

2. Guo, T.C.; Guo, W.W.; Larsen, L.E., "Medical Microwave Imagery on Inverse Scattering Approach", IEEE Transaction on Milimeter and Infra-Red, Special Publication IEEE #CH1917-4/83

3. Azimi, M.; Kak, A.C., "Distortion in Diffraction Imaging Caused by Multiple Scattering". IEEE Transaction Medical Imaging, MI:2, pp 176-195, 1983

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OC 6449
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	CD	215 WWJ8
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/2			

11. TITLE (Precede with Security Classification Code): (U) Mechanism of Response to Stress

12. SUBJECT AREAS: 0616 Physiology 0601 Biochemistry 0510 Psychology

13. START DATE: 76 07
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT/GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	3.0	377
85	3.0	490

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code): Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL: Top, F H Jr
d. TELEPHONE NUMBER (include area code): (202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS:
Division of Neuropsychiatry
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR: Meyerhoff, J L
d. TELEPHONE NUMBER (include area code): (202)-576-3559
f. NAME OF ASSOCIATE INVESTIGATOR (if available): Kant, G J
g. NAME OF ASSOCIATE INVESTIGATOR (if available): Walczak, D D

21. GENERAL USE: F I N A
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Stress; (U) Neurochemistry; (U) Neurotransmitters; (U)Post-traumatic epilepsy; (U)RAM III; (U)Lab Animals; (U)Rats

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To examine neurochemical mechanisms in adaptation to stress and continuous performance. To study neurochemical mechanisms in development of post-traumatic epilepsy, which occurs in 40% of soldiers suffering head wounds, despite anti-convulsant drugs. To provide database for interpretation of military clinical and field studies, and recommendations for prevention and/or treatment in soldiers.

24. (U) Analysis of neurochemical regulation of hormonal responses to acute and repeated stress, and during adaptation to chronic stress. Repeated electrical stimulation of the brain ("kindling") has been selected as the best animal model of post-traumatic epilepsy, because of similarities in time-course. Studies entail brain lesion and stimulation; measurement of neurotransmitters, neuropeptides, cyclic nucleotides and phosphorylation in specific brain regions.

25. (U) 83 10 - 84 09. Pituitary cyclic AMP response to stress varies with time of day, from a 15-fold rise just after "lights on" to no rise after "lights off"; a similar variation in response to i.v. corticotrophin releasing factor is found. Pituitary stalk transection blocks, and valium attenuates the rise in pituitary cyclic AMP induced by stress. Seizures induced by exposure to bicyclic organophosphates lowers pO_2 in hippocampus and amygdala: The prostaglandin polymer, PGB_X in increases survival rate in these rats. We found that levels of the excitatory peptide neurotransmitter N-acetyl aspartylglutamate (NAAG) are increased in entorhinal cortex following kindled seizures. The development of kindled seizures is markedly slowed by combined administration of muscarinic and nicotinic cholinergic blockers. For Technical Report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 215 Mechanism of Response to Stress
Investigators
Principal: Meyerhoff, J.L., M.D.
Associate: Kant, G.J., Ph.D.; Walczak, D.D., Ph.D., Cpt., MSC; Lynch, T.J., PhD.; Oleshansky, M.A.,M.D.,MAJ MC; Mougey, E.H., M.S.; Collins, D.R., B.S.; Pennington, L.L., B.S.

I. Neurochemical Mechanisms of Adaptation to Stress.:

Objectives:

Studies are conducted to evaluate neurochemical mechanisms of response to stress, brain injury and other factors which produce psychiatric incapacitation or brain syndromes pertinent to military medicine. Included are (1), studies of CNS regulation of neuroendocrine function in acute and repeated exposure to stressors, as well as in adaptation to chronic stress; (2), effects of stress or injury on CNS neurotransmitter turnover, neuropeptide chemistry and receptor function, as well as studies of neurochemical system interactions. These fundamental studies are intended to provide a database for interpretation of clinical psychoendocrine studies of stress in soldiers, and for understanding the mechanisms by which traumatic factors cause decrements in CNS function and performance.

Progress:

We have continued to investigate the role of pituitary cyclic AMP in the mechanism of release and synthesis of pituitary hormones following acute stress. Since we have shown that complete bilateral adrenalectomy abolished the stress-induced increase in pituitary cyclic AMP, experiments during the past year have focussed on the adrenal cortical (corticosterone system) and adrenal medullary hormones. In addition, the time course of the loss of pituitary responsiveness to stress following adrenalectomy has been studied and the pituitary response to various neurotransmitter agonists has been determined.

Epinephrine (adrenalin) is an adrenomedullary hormone that is released during stress and might be the factor responsible for the stress-induced pituitary cyclic AMP increase observed in intact rats. Injection of epinephrine in vivo to intact rats caused a dose-dependent increase in pituitary cyclic AMP levels. However, this response was not seen in adrenalectomized rats. Also splanchnic denervation which prevents activation of the adrenal medulla and blocks stress-induced increases in plasma epinephrine did not prevent stress-induced increases in pituitary cyclic AMP. Thus it appears unlikely that the loss of pituitary cyclic AMP response to stress in adrenalectomized animals is due to loss of adrenal epinephrine.

In animals adrenalectomized and stressed at various times following adrenalectomy, it was found that the loss of pituitary response to stress requires 48 hours to develop. Our previous studies were conducted with animals at least 7 days post adrenalectomy. Since adrenal epinephrine is removed from plasma very quickly, this is another piece of data that suggests epinephrine is not the factor responsible for stress-induced pituitary cyclic AMP increases. The time course data do correlate chronologically with changes in the CRF-ACTH-corticosterone system. CRF is the brain peptide that regulates ACTH (adrenocorticotropic hormone) release from the pituitary. ACTH, in turn, regulates the release of corticosterone from the adrenal cortex. Numerous feedback mechanisms seem to be involved in this system, e.g. corticosterone binds to brain to inhibit the release of ACTH. Levels of hypothalamic CRF have been reported to increase several days post adrenalectomy and pituitary CRF receptors have been reported to decrease several days following adrenalectomy.

To complement the in vivo studies described above, we set up an in vitro pituitary homogenate system . We found that CRF increased cyclic AMP accumulation in anterior pituitary homogenates in a dose-related manner from 3nM to 30 uM in both sham-operated and adrenalectomized rats. However, adrenalectomized rats had 30% lower basal pituitary adenylate cyclase activity than sham-operated controls. The dose response curve for CRF stimulation of pituitary adenylate cyclase from adrenalectomized rats was shifted significantly to the right as compared with sham operated rats. Maximal stimulation of pituitary adenylate cyclase was nearly double in control versus adrenalectomized rats.

These findings support our hypothesis that adrenalectomy, which removes the negative feedback of corticosterone on hypothalamic CRF production, is associated with the subsequent down-regulation of the pituitary CRF receptor/adenylate cyclase complex. As the stress-induced changes in pituitary cyclic AMP in vivo are related to the stress-induced release of CRF, the down-regulation of the CRF-stimulated adenylate cyclase in pituitary after adrenalectomy would appear to explain the failure of stress to increase pituitary cyclic AMP levels in vivo after adrenalectomy.

We believe that cyclic AMP in the pituitary is involved in the release and synthesis of ACTH and related hormones, under basal as well as stressful conditions.

We recently reported that repeated exposure to a stressor resulted in a diminished stress response following subsequent exposure to the same stressor as compared to stress response following initial exposure to the stressor. Stress response was assessed by plasma corticosterone and prolactin and pituitary cyclic AMP elevations immediately following a 15 min stressor exposure. Habituation in these experiments could be viewed as a desensitization to the stressor stimulus such that the perceived stress was lessened i.e. a behavioral adaption. Alternatively, repeated stress could cause changes in biochemical systems, e.g. changes in pituitary adenylate cyclase activity or prolactin synthesis, that might affect the amount of stress-released hormones. To distinguish between these possibilities, we exposed rats to a single stressor (footshock, immobilization, forced running, or control) for 10 days. On the 11th day rats were exposed either to the same stressor or a different stressor such that a complete cross stress design resulted. Plasma prolactin and pituitary cyclic AMP responses to each of the three stressors were diminished in rats that had previous exposure to the stressor applied immediately before sacrifice. However, repeated exposure to a one stressor did not habituate the rats to exposure to a different stressor. For example, previous experience with forced running diminished observed stress response to forced running but not to immobilization or footshock and vice versa. We conclude that habituation results from behavioral experience with a particular stressor rather than biochemical adaption resulting from repeated challenge to hormonal and neurochemical systems responsive to stress.

Much of our stressor work has focussed on responses to acute stress. We have now equipped and programmed a sustained stress environment for rats which should facilitate studies of the effects of chronic or sustained stress that might better model the effects of battlefield stressors. Rats are maintained in operant boxes and required to lever press for food. After habituation to the environment, footshock is added to the protocol. Rats can avoid shock by pulling a chain during a warning period or escape escalating shock by pulling the chain during shock. Rats live continuously in this environment for a period of days. Preliminary experiments demonstrate attenuated food intake and disrupted food patterning during 72 hours of the shock condition (study conducted at 6 shock onsets per hour). We plan to use this also as a model for clinical sleep deprivation studies (see Annual Report for Project 3E162777A879, Work Unit 044, Oct. 1984).

Future Objectives:

We plan to continue to study the mechanism of the pituitary cyclic AMP and other neuroendocrine and neuropeptide responses to stress. The interactions of neurotransmitters and hormones in modulating stress responses will be analysed using specific pharmacological blockers. The contribution of the adrenal gland will be further analysed by endocrine manipulations such as the use of dexamethasone or metyrapone to mimic or block the feedback effects of corticosterone. Further physiologic studies (i.e. amygdalar or septal lesions, etc.) will be initiated to understand limbic system regulation of pituitary responses. We shall extend the acute stress studies to include consideration of the chronic stress of continuous performance requirements. We will also continue to study the interaction between stress responses and pretreatment with pharmacological agents such as carbamate cholinesterase inhibitors, as well as with drugs known to enhance performance. We plan to assay brain noradrenergic receptors in stressed animals, and to study the effects of "stress hormones" on release of neurotransmitters. We plan to extend studies of sex differences in responses to acute and chronic stress.

We plan to examine the role of CRF in the pituitary cyclic AMP response to stress further. CRF will be injected iv to chronically catherized animals and the pituitary cyclic AMP response will be measured. Since corticosterone and presumably CRF vary over 24 hours, pituitary cyclic AMP and plasma hormone response to stress will be determined every 2 hours over 24 hours. Stress response will be measured in animals pretreated with antibodies to CRF, if these can be obtained.

During the next year we plan extensive experiments using the sustained stress model described above in which the effects of various stress intensities and shock durations will be tested with respect to effects on baseline and challenged neurochemical and neuroendocrine systems (e.g. neurotransmitter receptors, enzymes, turnover).

II Prevention of Post-Traumatic Epilepsy:

Objectives:

Studies are conducted to explore possible neurochemical mechanisms in the development of post-traumatic epilepsy. More than 50% of soldiers receiving penetrating missile injuries of the brain will subsequently develop post-traumatic epilepsy. Despite the development of anticonvulsant drugs and improvements in aseptic surgical technique, the incidence of post-traumatic epilepsy has not decreased since World War I. Understanding the biochemical mechanisms active during the latent period between the injury and the observation of clinical seizures is essential to developing rational preventive measures which might be initiated immediately post-injury. Based on our neurochemical findings, appropriate novel pharmacological interventions will be explored.

Progress:

In our project directed at post-traumatic epilepsy, we have succeeded in employing the "kindling" technique for producing epileptiform seizures. Kindling consists of repeated, intermittent low intensity electrical stimulation of the amygdala. This results in progressive changes in both electrographic and behavioral responses over several weeks, and culminates in a generalized seizure in response to an electrical stimulus which initially had produced no behavioral effect. Kindling seems a particularly good model of post-traumatic epilepsy because it permits biochemical study of seizure-prone brain tissue without requiring the use of seizure-inducing drugs. Moreover, the latent period seen in the kindling phenomenon is similar to the delay of seizure onset seen in post-traumatic epilepsy.

We have developed a new seizure model for use in neurochemical and pharmacological studies of seizures. The Suprathreshold Stimulation Model (STS) provides an easily-controlled acute and chronic seizure model that is intermediate in severity between Maximal Electroshock (MES) and sublethal Pentylenetetrazol (PTZ) models. Like MES, the STS seizure is produced by electrical stimulation of large areas of the brain. Unlike MES, the stimulation is applied directly to the cortex through extradural skull screws, thereby eliminating variations in stimulus parameters relating to variations in conductivity of extracranial tissue. The stimulation intensity is higher than the threshold for producing predominantly clonic seizures (hence "suprathreshold") but is far below that necessary to produce a maximal, primarily tonic seizure. Production of clonic seizures by submaximal stimulation in the normal MES paradigm is undesirable, as this often produces pronounced pain and discomfort in the rat by stimulation of peripheral sensory nerves. STS stimulation produces no pain at submaximal stimulation levels, since extracranial tissues with their attendant sensory nerves are bypassed, and current is delivered through the dura to the cortex. The type of seizure observed varies with the current intensity, and intensity can be "tuned" to elicit a seizure composed of clonic components that resemble those seen in fully generalized kindled seizures.

Seizure severity has been characterized as a function of stimulus intensity, stability of the response as a function of time has been examined, and the STS treatment is now an integral part of all neurochemical studies of kindling. The STS model provides a reliable and humane means of producing "kindled-type" clonic seizures acutely, and in a manner that can be repeated reliably over many days of stimulation. Use of this treatment group in neurochemical studies of kindling will provide a means of distinguishing between neurochemical changes potentially involved in the process of seizure generalization, and those that result from brain activation during seizures per se.

A pharmacological study of the role of cholinergic function in the development of generalized seizures in kindling has been completed. The study tested the hypothesis that both muscarinic and nicotini cholinergic function is involved in the progressive development of kindled seizures. Rats pretreated with a combination of Atropine (20 mg/kg) an Mecamylamine (10 mg/kg) exhibited increased latency to develop fully-generalized kindled seizures relative to saline-treated controls., defined as the number of stimulations required to elicit a stage five seizure. This effect was not observed in groups treated with either drug alone at the doses indicated. The increased latency to kindled was reflected in lower average seizure scores in the combination group, shorter duration of epileptiform afterdischarge (AD) over the kindling period, and shorter AD duration in combination groups even when stage five seizures were finally elicited. These data suggest that both muscarinic (Atropine-sensitive) and nicotinic (Mecamylamine-sensitive) neuronal systems are involved in the process of seizure generalization in kindling, and may be important targets for pharmacological intervention in other seizure conditions.

We have demonstrated that kindled seizures increase entorhinal cortical levels of the dipeptide, N-acetyl-aspartyl-glutamate (NAAG). Several laboratories have shown that NAAG is endogenous to brain. The reported findings of an uneven regional distribution in brain as well as reports that injection of kainic acid into the corpus striatum produces a 31% decrease in striatal levels suggests that NAAG is localized in neurons. Our finding that kindled seizures produce increases in NAAG in a specific brain region is consistent with body of literature suggesting that this dipeptide may be an excitatory neurotransmitter. Intra-hippocampal injection of NAAG produces seizures at doses similar to quisqualic acid, a glutamatic acid analog, and iontophoretically applied NAAG has potent excitatory effects on individual neurons in pre-pyriform cortex. Finally, NAAG has a high affinity for a subpopulation of glutamic acid receptors in brain tissue. The increase in entorhinal cortical NAAG produced by amygdaloid kindling suggests the involvement of this endogenous dipeptide in seizure mechanisms.

A relatively new organophosphate poison was tested for is potential to cause brain hypoxia in rats during the epileptic seizures which it induces. The compound is the bicyclic organophosphate, ethyl phosphatrioxabicyclo octane (EPTBO), and it is the chief combustion product in the burning of fire retarded plastics. Unlike the standard organophosphate poisons, its primary neurophysiological effect is antagonism of the inhibitory neurotransmitter, gamma aminobutyric acid (GABA). Rats were injected with an LD$_{50}$ dose of EPTBO and the partial pressure of oxygen (pO$_2$) in hippocampal brain tissue was monitored polarographically during the seizures which followed. The average response during the onset of each seizure was a 32% decrease in brain pO$_2$, runnning its course in 40 seconds, followed by a rebound increase of 42% over baseline pO$_2$, running its course in 2 minutes. Evidence so far shows this hypoxia also to be occurring in other structures of the brain, which is in striking constrast to the hyperoxia typically induced by another form of experimental epilepsy, kindling. At this time we are uncertain whether the transient hypoxia caused by EPTBO is due to seizure-induced respiratory embarrassment or to an increase in brain metabolism.

As part of ongoing research into the involvement of neurotransmitters and hormones in the development of epilepsy, our branch has directed some attention to the possible role of the gastrointestinal hormones vasoactive intestinal peptide (VIP) and cholecystokinin (CCK), which have recently also been found in the brain. In order to examine the levels of these hormone in epileptic rat brain it is necessary to sacrifice the animals in a manner which minimizes any post-mortem artifact. Humane methods of sacrifice include decapitation, microwave irradiation of the brain and rapid freezing with liquid nitrogen. In two complementary experiments we showed that the heat of microwave does not decrease brain CCK levels and that decapitation causes a postmortem increase on the order of 50%. While decapitation has been the method of choice for CCK assay in many laboratories, it appears that microwave yields values which more accurately reflect in vivo CCK levels. Studies of effects of physiological of psychological variables on regional brain CCK using decapitation as the mode of sacrifice would suffer from masking of effects, due to post-mortem artifact.

Future Objectives:

We plan to continue studies of neurochemical mechanism of development of seizure disorders following trauma, extending neuropeptide studies to include neurotensin, somatostatin, enkephalin, cholecystokinin, vasoactive intestinal peptide and motilin. We are planning further studies of novel pharmacologic interventions to prevent development of seizures following brain injury. Such studies will include the use of peptide agonists, and calcium channel blockers. Further studies are under way to determine if the effects of kindling on regional brain levels of TRH and NAAG are persistent, or of limited duration.

III. Presentations:

1. Walczak, D.D. and Meyerhoff, J.L. Supra-Threshold Stimulation (STS): an electrically-induced seizure model proposed as a control treatment in neurochemical studies of kindling. Annual meeting of the Federation of American Societies for Experimental Biology (FASEB), St. Louis, MO, March, 1984.

2. Kant, G.J. and Meyerhoff, J.L. Epinephrine increases Pituitary cyclic AMP *in vivo*. Annual Meeting of the Federation of American Societies for Experimental Biology (FASEB), St. Louis, MO, March, 1984.

IV. Publications:

1. Kant, G.J., Mougey, E.H., Pennington, L.L. and Meyerhoff, J.L. Graded footshock stress elevates pituitary cyclic AMP and plasma b-Endorphin, b LPH, corticosterone and prolactin. 1983. Life Sciences 33: 2657-2663.

2. Kant, G.J., Mougey, E.H., Collins, D.R., Wormley, C.B., Pennington, L.L. and Meyerhoff, J.L. Graded footshock stress elevates pituitary cyclic AMP and plasma b-endorphin, b-LPH, corticosterone and prolactin. 1983. Neuroscience abstracts, 9, 1124.

3. Nielsen, C.J. and Kant, G.J. The effects of footshock stress on glucocorticoid receptors in rat hippocampal cytosol. 1983. Neuroscience Abstracts, 9, 1123.

4. Mougey, E.H., Pennington, L.L., Gamble, W.L., Kant, G.J. and Meyerhoff, J.L. Dexamethasone does not suppress stress induced increases in pituitary cyclic AMP. 1983. Neuroscience Absracts, 9, 1123.

5. Meyerhoff, J.L., Kant, G.J., Nielsen, C.J., Mougey, E.H. and Pennington, L.L. Adrenalectomy abolishes stress-induced increase in pituitary cyclic AMP. 1983. Neuroscience Abstracts, 9, 1123.

6. Lynch, T.J. and Jackson, W.J. Ictal temperature transients in the kindled amygdaloid focus. 1983. Neuroscience Abstracts, 9: 144.

7. J. Meyerhoff, V. Bates, D. Walczak and M. Kubek. Elevated Thyrotropin Releasing Hormone (TRH) Associated with Partial and Fully Generalized Kindled Seizures. 1983. Epilepsy International Symposium, Abstracts 15, 327.

8. A. Sattin, J.L. Meyerhoff, G.P. Mueller and M.J. Kubek. Effects of Single and Repeated Electroconvulsive Shock (ECS) on Regional Brain Thyrotropin-Releasing Hormone (TRH) and Neurotensin (NT). 1983. Neuroscience Abstracts 9, 385.

9. M.J. Kubek, J.L. Meyerhoff, T.G. Hill, and A. Sattin. Effect of Thyroxine (T4) on Hypothalamic, Extrahypothalamic, and Pituitary Thyrotropin-Releasing Hormone (TRH). 1983. Neuroscience Abstracts 9, 393.

10. T.J. Cavanagh, T.G. Hill, W. Day, R.W. Roeske, J.M. Meyerhoff, and M.J. Kubek. Thyrotropin-Releasing Hormone (TRH): Degradation in Serum and Specific CNS Loci of the Rat. 1983. Neuroscience Abstracts 9, 1134.

12. D. Walczak, J.L. Meyerhoff, V.E. Bates, T. Lynch and M.J. Kubek. Effect of Partial and Fully Generalized kindled Seizures on Thyrotropin Releasing Hormone Levels in Specific Cortical and Subcortical Regions of Rat Brain. 1983. Neuroscience Abstracts 9, 485.

13. Landman-Roberts, L., Kant, G.J., Eggleston, T., Kenion, C.C., Driver, G.C. and Meyerhoff, J.L. Atropine Increases Sensitivity to Stress in Rats. 1983. Neuroscience Abstracts 9, 554.

14. Kant, G.J., Meyerhoff, J.L.,and Jarrard, L.E. Biochemical indices of reactivity and habituation in rats with hippocampal lesions. 1984. Pharmacol. Biochem. Behav. 20: 793-797.

15. Kant, G.J. and Meyerhoff, J.L. Epinephrine increases Pituitary cyclic AMP *in vivo*. 1984. Federation Proceedings, Abstracts, 43, 912.

16. Meyerhoff, J.L., Kant, G.J., Nielsen, C.J.,and Mougey, E.H. Adrenalectomy abolishes the stress-induced increase in pituitary cyclic AMP *in vivo*. 1984. Life Sciences, 34: 1959-1965.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA OC 6473	2. DATE OF SUMMARY 84 09 30	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY H. Termination	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	CD	216 WWJ5
b. CONTRIBUTING				
c. XXXXXXXXXX	STOG 82/83-6.2/2			

11. TITLE (Precede with Security Classification Code)
(U) Military Stress: Non-Invasive Monitoring of Health and Performance

12. SUBJECT AREAS
0510 Psychology, 0619 Stress Physiology

13. START DATE: 78 10
14. ESTIMATED COMPLETION DATE: 84 10
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	5.0	455
85	0.0	0

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER (include area code)
202 - 576-3551

20. PERFORMING ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS
Division of Neuropsychiatry
Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
GENSER, S G
d. TELEPHONE NUMBER (include area code)
301 - 427-5521
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
THORNE, D R
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
SING, H C

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Psychophysics; (U) Stress; (U) Performance; (U) Electrophysiology; (U) Psychophysiology; (U) Volunteers; (U) RAM III

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Development of non-invasive human psychophysiological monitoring technology in support of field studies of stress in military environments.

24. (U) Exploit advances in signal acquisition and processing technologies to investigate and develop means for recording and validating psychophysiologic measurement systems.

25. (U)
8310-8409 This unit is supplanted by a new work unit entitled, Military Stress: Biomedical Measurement ~~Systems (6.27.77.879 AS)~~. In FY 84, a system for processing of multiple measures was completed, and a new 9-channel ambulatory recording system was integrated into it. Design of a new analog sleep/activity monitor was validated. These systems were deployed in collaborations in Arkansas and Canada concerning temporal isolation, sleep deprivation and sustained operations. Refinement of the cognitive performance assessment battery emphasized conversion to a field deployable system. A Request for Proposal has been issued for a neuropsychological test battery (NTB), as the result of methodologic studies and recommendations of an expert panel. Contracts were let for application of pattern recognition methods to biomedical data series, and for digital sleep/activity monitors. A third contract is pending for a biomedical monitor/telemeter/thermal stress warning system for chemical defense field studies. Basic investigations under this unit have been achieved. The new work unit exploits this progress and broadens the applications in support of other departmental research projects. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sept 84.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

Project: 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURIES, AND HEALTH HAZARDS

Work Unit: 216: Military Stress: Non-invasive Monitoring of Health Performance

Investigators:

Principal: Sander G. Genser, M.D., COL MC

Associates: Daniel P. Redmond, M.D., LTC MC

David D. Thorne, Ph.D.

Helen C. Sing, M.S.

Problems and Objectives:

This work unit develops non-invasive laboratory and field deployable instruments to measure human sleep wakefullness cycles and levels of activity (actigraph), human physiological state (EKG, EEG, temperature), human cognitive performance Performance Assessment Battery (PAB), and Neuropsychological Test System (NTS), and human affect and level of activation Affect/Activiation Scale (MAS). The instruments developed are used in the departments studies in the laboratory and the field of sleep/wakefullness, circadian periodicity, and cognitive performance in sustained operations and long range deployments.

Progress:

Basic research and development of several biomedical monitoring systems was completed in FY84. The foremost of these involves the monitoring of human psychomotor activity (actigraphy) which will provide wear-and-forget methods for recording sleep/rest/work/activity data on individual subjects in the field and in the laboratory. Basic research efforts led to the successful specification and characterization of the signals to be recorded and a Technical Report on this subject is in preparation. An ambulatory tape recorder actigraph system was designed in-house and deployed in several collaborative studies: 1) University of Arkansas - studies of temporal isolation of human subjects, completing work on ambulatory blood presssure monitoring (Publication #1); 2) Canadian Defense and Civil Institute of Environmental Medicine (DCIEM) - studies of sleep deprivation and napping; 3) 1st Armored Cavalry Division, Ft. Hood - studies of command post exercises in armored battalions; 4) CHACE II - a study in collaboration with DCIEM of sustained operations in casualty management in a chemical environment. In addition, two manufacturing contracts were negotiated for production of digitized, miniaturized, wrist-worn actigraphs. One system is a simplified, rugged, and suitable for field use, and the other system is a sophisticated, micro-processor based, and suitable of laboratory use. Delivery of 50 units of each system is scheduled for April 85. Further, laboratory systems for data retrieval and processing these ambulatory monitors were developed in the laboratory. A system providing concurrent processing of actigraph and EKG data in 24 hour long series was designed and implemented. In this connection, a three year contract was awarded to George Washington University for the application of modern pattern recognition technology to activity and heart rate data series for the purposes of deriving algorithms to detect from actigraph and EKG data sleep-wakefullness and other physiological changes of state. A review of temperature recording technology, especially regarding the swallowable temperature pill, coupled with the need to develope a telemetered physiologic data acquisition system in free-roaming subjects in chemical protective gear, led to the specification of the Chemical Defense User Safety System (CDUSS). A request for proposal was generated, and a source selection board convened. Delivery of the prototype system of this multi-channel data acquisition and thermal stress monitor is anticipated by the end of FY85.

The Performance Assessment Battery (PAB) has been modified to include two performance tasks specifically designed for use in a collaborative study with the Department of Medicine, WRAIR on the psycho-pharmacokinetics of atropine. Specifically, automated versions of a time estimation task and a spatial neglect assessment task were incorporated. A performance index (Throughput) has been developed. It is generalizable, and comparable across many different tasks and settings. It is sensititive to the effects of fatigue but relatively insensitive to inter-subject variability in speed/accuracy. The PAB has been requested by and supplied to many individuals, agencies, and institutions in the US, the UK, and Isreal. The PAB has been used in collaboration with the USAARL in assessing the performance effects of heat stress from wearing MOPP IV gear in helicopter pilots. The PAB has been used in collaboration with AFIP in assessing the performance effects of hypobaric exposure to persons with sickle cell trait. Preliminary design specifications for the proposed field-deployable performance assessment battery (FPAB) have been completed. Host hardware, operating system, implementation language have been specified and obtained. Preliminary specifications have been developed for architecture of the FPAB application system and its integration into the host ADP environment.

Basic research work is being conducted on the constituent elements of the neuropsychological test system (NTS). This research has focused upon comparing automated and standard (human tester) presentation of the digit span. The current study compares the presentation of an auditory automated version of the digit span with the standard presentation. Measures of the subjects response to the task include accuracy data, reaction times, mood/activation data, responses on a mood scale, and investigator observation. A request for proposal (RFQ) has been written for an automated neuropsychological test system (NTS). This document is a template for an NTS specifying anatomic areas and psychological functions to be tested. Also delineated are the technological requirements, including the operating system, and the meta-language to be used. A mission element needs statement (MENS) has been written and approved. An appendix L for the required ADP has been written and submitted to OTSG for approval. Upon approval of the appendix, the RFQ will be advertised. When developed, the NTS will provide an automated battery to provide quantitative information on cognition.

Publications:

1. Halberg, F, and D.P. Redmond Chronobiology of Human Blood Pressure

in the light of static (room-restricted) automated monitoring. Chronobiologia 11(1984)25-54.

Presentations: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OB 6448	2. DATE OF SUMMARY 841001	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 831001	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AG	217 WWMA
b. [illegible]				
c. [illegible]	STOG 82/83-6.2/3			

11. TITLE *(Precede with Security Classification Code)*
(U) Pharmacology of Candidate Anti-Parasitic Drugs

12. SUBJECT AREAS
0603 Biology 0703 Organic Chemistry 0615 Pharmacology

13. START DATE 6807	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORK YEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		84	3.0	492
c. TYPE	d. AMOUNT	85	3.0	518
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Experimental Therapeutics
b. ADDRESS *(include zip code)* Washington, DC 20307-5100	b. ADDRESS Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Reid, W A
d. TELEPHONE NUMBER *(include area code)* (202)-576-3551	d. TELEPHONE NUMBER *(include area code)* (301)-427-5029
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* LOWENSOHN, H S g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* WILLET, G P

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U)Parasitic Diseases; (U)Drug Development; (U)Pharmacology; (U)Chemistry; (U)Biology; (U)RAD I; (U)Lab Animals; (U)Dogs

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23.(U)To investigate basic chemical, biological, and pharmacological aspects in drug development for use against parasitic diseases of military importance.

24.(U)Chemicals are synthesized, characterized, and analyzed for study, culture systems and animal models of parasitic diseases are developed and used, drug delivery systems and animal models are developed, all to study the efficacy and toxicology of potential drugs. 25.(U)-840) Work continued on the development of a chemically defined culture medium for the in vitro cultivation of P. falciparum malaria. Studies were undertaken to evaluate parasite nutritional requirements in a special formulation of RPMI 1640 modified to amino acid and vitamin content and supplemented with oleic acid and bovine albumin. The modified medium supported parasite growth for up to 18 days. Further studies were conducted on the supplemental requirements for lipids and fatty acids in growth media. Procedures were established for the determination of quinine and its metabolites in bile following intravenous infusion of quinine. This establishes a definitive capability for sequentially quantitating quinine and its metabolites in bile. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

Project 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 217 Pharmacology of Candidate Anti-Parasitic Drugs

Investigators:
Principal: LTC Willis A. Reid, Jr., Ph.D.
Associate: H.S. Lowensohn, Ph.D., G.P. Willet
Assistant: SP5 L. Gerena, D.D. Wilson

1. Description.

The purpose of this work is to investigate basic chemical, biological, and pharmacological aspects of candidate new drugs in development for use against parasitic diseases of military importance.

2. Progress.

Initial studies on the absorption, distribution, metabolism and excretion of the antileishmanial drug WR 6026·HCl have been completed. An initial study of pharmacokinetics in patients receiving 60 mg single doses has been completed. Since this drug possesses anticyanide activity, it is also of interest to Project 995 (Preclinical Studies of Anti-Chemical Warfare Drugs). Methods have been developed to measure WR 6026, WR 238,605, artesunate, and dihydroqinghaosu (a breakdown product of artesunate) in biological samples. Absorption, pharmacokinetic and metabolism studies of the candidate antimalarial drugs WR 238,605 and artesunate are now in progress utilizing these new analytical techniques. WR 238,605 has been selected for development based on characteristics superior to primaquine in the rhesus monkey model.

A sensitive _in vitro_ technique for measuring the ability of some of these compounds to produce methemoglobin has been developed. This is an important side effect that must be considered in development.

In other work, a canine bile model has been developed to study the enterohepatic circulation of quinine and related compounds. The recent focus of this work has been to measure quinine and its metabolites in bile for the purpose of explaining the kinetics of this drug. This information can serve as a useful model for other antiparasitic drugs that display similar kinetics. As part of this work, specific chemical methods have been developed to measure quinine and tetracycline (used simultaneously in malaria treatment) by high pressure liquid chromatography. These methods are being used to study the pharmacokinetics of quinine in patients with falciparum malaria and to study quinine-tetracycline interactions in patient plasma.

Efforts continue to develop a model system of defined medium to culture *P. falciparum*. Once completed, this model will permit a variety of new *in vitro* experiments to define the nutritional requirements of the parasite and permit more rapid initial screens for drug activity.

3. Future Work.

Pharmacokinetic and metabolism studies of WR 6026, WR 238,605, and artesunate will continue in order to support the future development of these drugs. The work on the canine enterohepatic circulation model has been suspended. The quinine and tetracycline work in human samples will continue. Efforts to develop a completely defined growth medium for *P. falciparum* will continue.

4. Publications.

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Theoharides, A.D., H. Chung, and H. Velazquez. Metabolism of a potential 8-aminoquinoline antileishmanial drug in rat liver microsomes. Biochem. Pharmacol. (in press).

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY
1. AGENCY ACCESSION: DA OA 6449
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AF	218 WWG7
b. CONTRIBUTING				
c. XXXXXXXXXX	STOG 82/83 -	6.2/3		

11. TITLE (Precede with Security Classification Code)
(U) Immunological Mechanisms in Microbial Infections

12. SUBJECT AREAS
0613 Microbiology 0605 Clinical Medicine

13. START DATE: 62 08
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	4.0	415
85	4.0	416

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code): Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL: TOP, F H JR
d. TELEPHONE NUMBER (include area code): (202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME: Division of CD&I
b. ADDRESS:
Walter Reed Army Institute of Research
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR: Hockmeyer, W T
d. TELEPHONE NUMBER (include area code): (202)-576-3544
f. NAME OF ASSOCIATE INVESTIGATOR (if available): Crawford, R M
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE: FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Lab Animals; (U) Mice; (U) RAMI; (U) Antibodies; (U) Antigens; (U) Protozoa; (U) Immunoassay; (U) Leishmania

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23 (U) The objective is to find suitable antigens that can be used to develop vaccines for parasitic diseases that pose a significant threat to military operations in endemic areas.

24 (U) The approaches used for these studies involve development of immunoprecipitation and electroblotting techniques to characterize surface antigens which in the presence of polyclonal human immune serum or monoclonal antibodies plus C kill promastigotes in vitro.

25 (U) 83 10 - 84 09 Surface reactive monoclonal antibodies (MAbs) are being continually developed and screened for parasitocidal effects against L. donovani promastigote in the C mediated assay. Those demonstrating cidal activity are used as probes to characterize surface proteins immunochemically by SDS-PAGE Western Blot or precipitation reactions and ultimately to screen DNA recombinant libraries. Parasitocidal MAbs have been shown to recognize both single proteins (approximately 30,000 kd and 80,000 kd) by Western Blot as well as multilple proteins (30,000 kd thru 80,000 kd) by precipitation. The 30,000 kd protein may be a maturation protein since it appears later in culture similar to infectivity. A pool of these MAbs is now being used to screen DNA libraries for clones producing parasite surface proteins. Clone products will then be used in immunization trials to ascertain their suitability for a protective vaccine against L. donovani. For technical report see Walter Reed Army Institute of Research Annual Report, 1 Oct 83 - 30 Sep 84.

PROJECT 3M161102BS10 RESEARCH ON MILITARY DISEASES, INJURY AND HEALTH HAZARDS

Work Unit: 218 Immunological Mechanisms in Microbial Infections

Investigators:

Principals: MAJ W. Ripley Ballou, MC
MAJ Robert M. Crawford, MSC
LTC Wayne T. Hockmeyer, MSC

Associates: Mr. Rufus Gore
Mr. Joseph Williams

Problems and Objective:

Leishmaniasis: Promastigotes killing by complement dependant factors in serum has been an area of research pursued by our laboratory over the past 18 months. Our data confirm that the mechanism of nonimmune serum killing is largely dependant upon the alternate complement pathway. We extended these observations to include examination of the promastigote killing activity of high titre visceral leishmaniasis immune serum. IgG was purified from immune serum. Promastigotes were incubated at 37°C in a complement source to which heat inactivated normal serum or immune IgG was added. The effect of C2 deficient serum and serum depleted of factor B upon promastigotes was investigated. Promastigote viability was determined by differential fluorescent microscopy using the fluorescent dyes ethidium bromide and fluoroscein diacetate as markers of nonviable and viable promastigotes respectively. In contrast to nonimmune serum which kills primarily by the alternate pathway, immune IgG activated complement by the classical pathway to kill promastigotes even in the presence of very dilute complement. Human immune IgG will also activate guinea pig complement to kill promastigotes. In an attempt to identify surface antigens which may be important in this mechanism of parasite killing we have obtained and purified a number of mouse monoclonal antibodies (mabs) which recognize certain surface proteins of promastigotes. We are currently screening these mabs in the complement mediated killing assay for activity against long term culture forms of *L. donovani* promastigotes.

Promastigotes derived from sandflies are the infective form of the parasite in naturally occuring disease. There may be antigenic differences between these forms and promastigotes maintained in long term cultures. We have studied the effect of nonimmune serum on sandfly derived promastigotes in the complement mediated killing assay and have shown that these parasites are considerably more resistant to normal serum than long term forms. Immune IgG augments the activity of serum against these parasites but not to the extent seen with long term culture forms. The basis for this important observation is under current investigation.

Mabs can be used to identify bacterial clones producing antigens recognized by those mabs. A genomic library has been prepared with DNA obtained from *L. donovani* promastigotes and is being screened with a pool of five mabs which recognize specific promastigote surface proteins by Western blotting and immunoprecipitation. Preliminary data suggest that specific antigens emerge on the surface coat of stationary phase infective promastigotes. It is possible that mabs which activate complement to kill promastigotes may identify important leishmanial antigens such as those involved with maturation and perhaps infectivity of the promastigotes.

Recommendations:

There remain formidable problems with visceral leishmaniasis in regards to the identification of antigens which may be protective in this disease. In view of the identification of human antibodies which can kill the parasite a continued search for monoclonal antibodies should be pursued. An alternate method of screening prospective antigens for activity in addition to their ability to elicit leishmaniacidal antibodies should be developed in light of evidence that cellular immunity is important in protection and the finding that sandfly promastigotes appear to be resistant to serum killing. Continued examination of the immunochemistry of prospective antigens will play a key role in this vaccine development effort.

Formal Presentations:

1. Ballou, W.R., Killing of L. donovani promastigotes by immune serum. Washington Helminthological Society, March 1984.

2. Ballou, W.R., Crawford, R.M., and Hockmeyer, W.T. Identification of possible protective Leishmania donovani surface antigens. ASBC/AAI Meeting, St. Louis, MO. June 1984.

Published Abstracts:

1. Ballou, W.R., Crawford, R.M., and Hockmeyer, W.T. 1984. Identification of possible protective Leishmania donovani surface antigens. Fed. Proc. 43:2063 (June).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OA 6464
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	BD	220 WWI4
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 82/83-6.2/4			

11. TITLE (Precede with Security Classification Code)
(U) Pathogenesis of Renal Disease of Military Importance

12. SUBJECT AREAS
0616 Physiology, 0605 Clinical Medicine, 0619 Stress Physiology

13. START DATE: 54 09
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	13.0	652
85	13.0	681

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code): Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL: TOP F H JR
d. TELEPHONE NUMBER (include area code): (202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME: Division of Medicine
Walter Reed Army Institute of Research
b. ADDRESS: Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR: Johnson, J P
d. TELEPHONE NUMBER (include area code): (202)-576-2386
f. NAME OF ASSOCIATE INVESTIGATOR (if available): Butkus, D E
g. NAME OF ASSOCIATE INVESTIGATOR (if available): Wiesmann, W P

21. GENERAL USE: FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Renal Failure; (U) Renal Hemodynamics; (U) Heat Stress; (U) Shock; (U) Fluid and Solute Homeostasis; (U) Kidney Function; (U) Lab animals; (U) Dogs; (U) Rats (U) Volunteers; (U) RAM II:

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23.(U) To investigate methods for maintaining fluid, electrolyte and hemodynamic homeostasis in response to disease, injury and environmental stress of military significance such as shock, trauma, infectious disease, heat stress, gastrointestinal disorders and nephrotoxic drugs in order to provide rational basis for prevention and treatment of renal failure.

24.(U) Clearance methods, isolated tubule perfusion, membrane transport, intracellular microelectrodes, optical imaging techniques, tissue culture, enzyme kinetics, chromatography and isotope dilution.

25. (U) 83 10 - 84 09

The Department has developed five (5) animal models of acute renal failure. These models have been developed to study mechanisms and therapy of varying severity ARF due to 1) pure toxic injury; 2) pure ischemic injury and 3) combined toxic/ischemic damage. Currently used for study of protective or therapeutic maneuvers are: 1) renal artery clamp in the rat; 2) myoglobinuric ARF in the rat; 3) gentamicin nephrotoxicity in dog and rat; 4) combined hemorrhage and myoglobin in the dog; 5) cis-platinum nephrotoxicity in the rat. Studies have demonstrated an effect of urine pH and flow rate on ameliorating toxic and ischemic ARF and have demonstrated a substantial role for tubular obstruction in toxic ARF. Hemorrhage insufficient to cause ARF markedly potentiates ARF associated with myoglobin. An in vitro renal tubule preparation has been developed and analyzed by Diode-Array spectroscopy to examine the effect of toxins and hypoxia on renal cell metabolism and the generation of free-radicals. This system is being used to study the efficacy of metabolic agents to prevent or reverse cell death due to hypoxia or free radical formation. Agents useful in vitro will be applied to the animal models which have been developed. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project: 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 220 Pathogenesis of Renal Disease of Military Importance

Investigators:

Principal:	LTC(P) John P. Johnson, MC
Associates:	COL Donald E. Butkus, MC
	LTC William P. Wiesmann
	LTC Cristobal G. Duarte, MC
	MAJ James L. Atkins
	Richard S. Fisher, Ph.D.
	Mr. John A. Gagnon
	Mr. James S. McNeil

Problems and Objectives

The major effort of this Department has been directed toward the problem of acute renal failure in the combat casualty. In this setting, acute renal failure is associated with a persistant high mortality in spite of the institution of aggressive recussitation measures and close support to the battlefield with hemodialysis and peritoneal dialysis. Although the incidence of renal failure per seriously wounded decreased during the Vietnam era in response to more rapid volume expansion and triage, the mortality rate for established renal failure remained in excess of 50%. In a setting of multiple injuries and sepsis, a population with a high mortality per se, the presence of renal failure identified a particularly high risk group of patients. The inference that acute renal failure participates in this high mortality is maintained despite the inability of hemodialysis, as performed, to alter this risk. We believe that efforts to ameliorate or reverse renal failure should result in improved survival.

Major research in this field in the past has concentrated on the vasomotor events surrounding the initiation of renal failure. However, interventions during this period beyond reversal of shock in the septic or hypotensive patient are extremely difficult and potentially associated with deliterious systemic side effects. Moreover, maneuvers designed to maintain renal perfusion during initiation phases of experimental acute renal failure have met with variably poor results even in the face of maintaining total organ perfusion. We have, therefore, directed our research along lines more likely to provide therapeutic agents which may be of value not only to the kidney but also to the whole organism in a shock state. Current theory of the pathogenesis of acute renal failure holds that following an initiation phase dominated in all models by decreased perfusion, renal failure is maintained as a result of cellular anoxic or toxic injury. Our approach is to direct therapy towards maintenance or return of metabolic integrity in cells of the damaged organ. The experimental approach is twofold: 1) to study the effect of rescue agents on renal cell metabolism and survival in vitro and, 2) apply successful agents to whole animal models in-vivo to determine efficacy and design therapeutic strategies.

Progress

Considerable evidence of vasomotor changes during the first twelve hours following the initial insult in ischemic or toxic renal failure have suggested that pretreatment or very early intervention designed to maintain solute diuresis, alkaline urine pH or decreased afferent arteriolar resistance may modify the course of ARF. In particular, inhibition of the vasodilating prostaglandin system has been shown to accentuate the course of a wide variety of experimental ARF models (see below). While the course of ARF may be influenced in its initiation, it is likely that most ARF in combat casualties will be recognized during its maintenance phase. Pretreatment and extremely early use of vasodilators on the battlefield are not practicable solutions. We have, therefore, initiated studies to develop reliable ways to identify treatment modalities for maintenance of cellular integrity at the earliest and latest reversible stages. For the last two years, we have concentrated on efforts to maintain cellular ATP synthesis and mitochondrial function based on studies derived from RBC survival, ischemic damage to CNS, myocardium and kidney. Both cultured epithelial cells and suspensions of renal cortical tubules have been studied in-vitro to evaluate agents which will maintain cellular ATP stores and prevent cell death in the presence of varying degrees of hypoxia. Two potentially valuable agents have been tentatively identified (see below). In order to study these agents effectively we have also continued to develop simple animal models to cover the spectrum of ischemic and toxic ARF.

Studies in Ischemic and Combined Toxic-Ischemic Renal Failure

After several unsuccessful attempts in our laboratory to develop a reproducible ischemic model of ARF, our most recent effort being the combining of hemorrhage hypotension, partial occlusion of the aorta and hypoxia (see Annual Report, 1 Oct 82 -

30 Sept 83), we concluded that development of an ischemic model of ARF, short or total renal artery occlusion, was not probable or profitable. We therefore instituted a clamp model of ARF and sought to develop combined models of hemorrhge and myoglobin induced ARF in dog and rat.

Total occlusion of renal arteries, either unilateral or bilateral, will produce graded renal failure in the rat and this is dependent on time of occlusion. Studies in our laboratory demonstrate, as others have shown, that complete occlusion for less than 20 minutes produces mild non-oliguric ARF, while occlusion for one hour or longer results in renal infarction, total ARF and death of the animals. With 40 minutes of occlusion, rats develop substantial oliguric renal failure from which they eventually recover. This represents an easily achieved animal model for the study of therapeutic interventions before or after the ischemic insult. Degree of attenuation of ARF can be judged by comparing rate of decline of GFR, peak serum creatinines and recovery time. In an initial study of the effect of urine pH on ARF, control animals were compared to rats receiving either NaCL, $NaHCO_3$, or diamox (acetazolamide, a carbonic anhydrase inhibitor). These maneuvers produce either solute diuresis with no change in pH, solute diuresis with systemic and urinary alkalosis, and no solute diuresis with systemic acidosis and urinary alkalosis respectively. The presence of alkaline urine, with or without systemic alkalosis or solute diuresis, was found to ameliorate the severity of clamp ARF as judged by peak serum creatinine and recovery time. Tubular pH elevations should act primarily to prevent formation of intratubular casts and these results suggest a role for tubular obstruction in the maintenance of pure ischemic ARF. Besides demonstrating that our model is capable of manipulation, these results have significance for early adaptation to clinical trials as a number of anecdotal clinical observations suggest a value for alkalinizing urine early in post-traumatic ARF. It is noteworthy that urinary alkalinization may likely potentiate the effects of experimental aminoglycoside ARF, suggesting that such clinical studies need careful definition of patient populations.

While hemorrhagic hypotension is a common feature of post-traumatic ARF in man, ARF cannot be reliably produced in animals by this maneuver. We sought to determine whether ARF, similar to that seen after trauma, could be produced in conscious dogs using combinations of sublethal hemorrhage and myoglobin (myo) infusion.

All dogs were given a single dose of furosemide (2 mg/kg) and three days of low salt diet and NH_4CL drinking water to produce aciduria. Hemorrhage, to 50 mM Hg mean arterial pressure for two hours, was performed through femoral artery catheters.

ARF was assessed by serial changes in serum creatinine 1-4 days after insult, and when present, was accompanied by increases and decreases in fractional sodium excretion and urinary osmolality, respectively. Low dose myo (0.5g/Kg) produced mild, reversible ARF in half the animals, while high dose myo (1.0g/Kg) produced more severe, but still reversible ARF in all animals. Hemorrhage alone had no effect on renal function, while hemorrhage and low dose myo produced ARF similar to that seen in high dose myo alone. Hemorrhge plus high dose myo produced severe ARF in which recovery did not occur. We conclude that hemorrhge to a degree insufficient to cause ARF alone can potentiate the nephrotoxic effects of myoglobin, and demonstrate that graded levels of ARF can be produced in unanesthetized animals by these methods. A model capable of producing ARF of varying severity should prove useful in the evaluation of attenuating maneuvers in post-traumatic ARF.

We also studied the effects of hemorrhage, myoglobin and an additional stress, prostaglandin inhibition, on the genesis of ARF in the rat. In these experiments no attempt was made to lower urine pH by NH_4CL loading as these volume depleted animals were already excreting an acid urine. Interestingly, when a diet change inadvertently instituted by Vet Resources resulted in a more alkaline diet, the development of alkaline urine pH blocked myoglobin induced renal failure in some animals. Rats received either no treatment (controls), hemorrhage to reduce blood volume by 25%, Indomethecin (5 mg/kg), myoglobin (0.6 g/kg) or a combination. Neither hemorrhage, indo or myoglobin alone caused significant ARF. As in the dog, however, myoglobin potentiated the effect of both hemorrhage and indomethecin to produce ARF. The most significant effect was seen with indo and myo together. Interestingly, and in contrast to our experience with dog, hemorrhage had no effect when added to the combined indo and myo insult. When kidneys from these animals were examined pathologically, a profound effect on inner medullary cast formation and tissue swelling was noted which correlated linearly with degree of ARF. These observations suggest an important element of intraluminal obstruction in the pathogenesis of this model of ARF and in conjunction with the observations on urinary pH suggest a real benefit from a simple maneuver such as alkalinizing urine as a prophylactic or possibly therapeutic modality in post-traumatic ARF.

Studies in Non-Ischemic Models of Renal Failure

Since a significant portion of ARF in the combat casualty apparently arises in consequence of sepsis or antibiotic toxicity, we have continued to maintain and study models of pure toxic

ARF. A model of gentamicin induced ARF has been well characterized in this laboratory including the effects of prostaglandin inhibitors in this form of ARF (see Annual Report, 1 Oct 82 - 30 Sept 83). In an initial attempt to examine intrarenal protective mechanisms we have studied the effect of unilateral ureteral occlusion on gentamicin nephrotoxicity in the rat. Paired groups of rats received either unilateral ureteral occlusion or sham operation prior to receiving a nephrotoxic dose of gentamicin (40 mg/kg). Animals injected with gentamicin following ureteral occlusion did not exhibit an increase in serum creatinine while animals that were sham operated had a continued course of ARF for seven days after stopping gentamicin therapy. Clinically this means that gentamicin nephrotoxicity is blunted and that the presence of a single obstructed kidney in the patient receiving this agent is a definite indication for relief of obstruction even if the other kidney is unobstructed. Additionally, these observations suggest some protective effect from the obstructed kidney upon the unobstructed side. These observations are in accord with several recent experimental studies of the difficulty of producing ARF following uninephrectomy. It would appear that the compensatory hypertrophy initiated by unilateral nephrectomy or, in this case, obstruction, triggers some mechanism which results in relative protection from ARF.

Further study of the renal failure induced by cis-platinum (CDDP), a heavy metal compound used as an anti-neoplastic agent, has resulted in the description of the relationship between morphological and functional alterations. Induction of CDPP ARF was associated with morphological alterations throughout the proximal tubule. The severity of injury in early proximal tubule (P_1 and P_2) showed a significant correlation with degree of functional impairment. The degree of injury in P_3 did not correlate with function even at late time periods when necrosis became widespread. Agents capable of ameliorating CDPP-induced ARF demonstrated both functional (see Annual Report, 1 Oct 82 - 30 Sept 83) and anatomic protection with a reduction of tubular injury, particularly in P_1 and P_2. P_1 and P_2 contain the most metabolically active cells of the proximal tubule and these results demonstrate that anatomic abnormalities of the major transporting segments correlate with decrease in GFR.

In-Vitro Studies

In all forms of ARF there is a decrease in renal excretory functions. The decline in renal function can also be secondary to direct tubular damage. Recent evidence suggests that in ischemic renal failure, tubular leakage and obstruction can account for most of the decrease in renal function, with close to 100% of the filtered fluid leaking from the nephron by the end of the proximal tubule. In-vivo models are difficult to manipulate due to large regional differences in blood flow within the cortex. We have, therefore, chosen an in-vitro model to study, a suspension of cortical tubules. Rat kidneys are perfused in-situ with a well-oxygenated room temperature Ringer's solution containing 1 gr%Ficoll. As the kidneys pale, the kidneys are perfused with collagenase, broken apart, washed and resuspended in Ringer's with a standard protein concentration. The procedure requires 45 minutes and the yield is sufficient to run two simultaneous experiments. As evidence of viability, these tubules uniformly excluded the vital dye, Trypan-blue. The tubules were studied in water jacketed glass chambers held at 37° and oxygen tension of the solution could be critically monitored using an 0_2 electrode. Tungsten light was passed through the suspension and the transmitted light was collected on a rapid scanning diode array. With this method, the oxidation state of the cytochromes could be monitored at one second intervals. Oxidation state of cytachrome AA_3 correlated well with oxygen delivery. When 0_2 delivery to the suspension is stopped, the 0_2 consumption rate of tubules was so brisk that virtually all of the 0_2 was extracted from the tubules within two minutes. This rate of 0_2 consumption was markedly slowed in the presence of ouabain, indicating that a major portion of the 0_2 consumption is related to the function of the Na-K ATPase as it is in-vivo. Decrease in 0_2 delivery resulted in progressive reduction of the cytochromes and the NAD-FAD peaks. After one hour of anoxia all tubules took up trypan-blue. Since dye uptake is difficult to quantitate, cellular necrosis was also correlated with release of cellular enzymes, LDH and Leucine Amino peptidase (LAP). Release of LAP and LDH into the suspension correlated well with reduction of cytochrome AA_3 and with ATP levels (measured by HPLC) which fell to 10-20% of control levels with anoxia.

Initial studies of tubule suspensions with purine analogs (see Annual Report 1 Oct 82 - 30 Sept 83) gave good evidence of maintenance of ATP levels during anoxia. Recently we have studied the effects of Ribose. Ribose is essential to anaerobic ATP synthesis by both de nova and salvage pathways and has been shown to maintain cellular function and limit damage in ischemic in myocardium. It has distinct advantages as a therapeutic agent compared to adenosine, which is a renal vasoconstrictor, in that ribose is harmless and systemic concentrations in the mM range may be attained. In the presence of 10 mM ribose during anoxic periods up to 20 minutes, the release of LDH and LAP into the media are markedly blunted and ATP levels are higher than in cells incubated with glucose. The specific activity of ^{14}C-ATP from

^{14}-C adenosine in solution is substantially higher indicating enhancement of ATP synthesis during anoxia. These results suggest that a simple, nontoxic sugar may limit cellular damage by stimulating ATP salvage pathways after anoxia. Ribose, like adenosine and Mg-ATP, may have promise as a possible rescue agent from anoxic insults.

Basic Scientific Research

The department continues to conduct basic research for methods development to be used in future mission-related research. Studies of lipid synthetic pathways and their response to normal regulatory pathways have led to techniques for use of cultured cells as models for membrane related events. LTC Wiesmann has continued his collaborative efforts wih AFRIMS on purine pathways in malarially infested RBCs with a view to targeted drug therapy. Studies of basic ionic permeability properties of epithelial tissues will lead to a definition of membrane properties subject to measurement during toxic or anoxic stress.

Future Plans and Recommendations

Further studies are planned with the in-vitro tubule preparation to determine whether any discernable biochemical changes such as enzyme release or cellular pH corresond to the onset of irreversible cell damage. Studies with purine analogs and ribose-1-PO$_4$ are planned in-vitro to determine optimal concentrations and under what conditions - pre-incubation or addition with reoxygenation - cellular ATP lvels are most enhanced. Projected collaboration with AFRI will study the generation of free radicals in clamp ARF and the ability of xanthine oxidase inhibitors to block free radical formation in-vivo and in-vitro. Initial studies of urine pH adjustments following mixed toxic ischemic injury will be undertaken as well as initial dose/response intervals for the use of ribose in clamp ARF. We have also undertaken to study collaboratively the actions of an agent developed in the ONR stress program which is said to preserve mitochondrial function following ischemia. Studies of basic mechanisms of cell-membrane function would be enhanced by development of techniques of or acquisition of an investigator familiar with vesicle studies.

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Butkus, D.E.: Persistant High Mortality in Acute Renal Failure. Arch Int Med 143:209-212, 1983.

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Dadonna, P., Wiesmann, W.P. and Webster, H.K.: Human Malaria Parasite Adenosine Deaminase. J. Biol. Chem. 259:1472-1475, 1984.

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Tang, L., Schoomaker, E. and Wiesmann, W.P.: Cholinergic Agnosts Stimulate Calcium Uptake and cGMP Formation in Human Erythrocytes. Biochim et Biophys Acta. 772:235-238, 1984.

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DAOG 6466

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION ~~DA OG 6466~~	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
PRIMARY	61102A	3M161102BS10	AI	222 WWP2
~~CONTRIBUTING~~				
~~CONTRIBUTING~~	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Histopathologic Manifestation of Military Diseases and Injuries

12. SUBJECT AREAS
0603 Biology 0613 Microbiology

13. START DATE: 63 08
14. ESTIMATED COMPLETION DATE: Cont
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
DATE EFFECTIVE
EXPIRATION
CONTRACT/GRANT NUMBER
TYPE
d. AMOUNT
KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	1.0	312
85	1.0	326

19. RESPONSIBLE DOD ORGANIZATION
NAME Walter Reed Army Institute of Research
ADDRESS (include zip code)
Washington, DC 20307-5100
NAME OF RESPONSIBLE INDIVIDUAL
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TELEPHONE NUMBER (include area code)
202-576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
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b. ADDRESS
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
Tseng, J
d. TELEPHONE NUMBER (include area code)
202-576-2024
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Gut Lamina Propria; (U) IgA Plasma Cells; (U) Peyer's Patches; (U) Migration and Differentiation; (U) Lab animals; (U) Mice; (U) RAM I (U) Monkeys

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23 (U) Enteric diseases are important medical problems constantly affecting army personnel. To determine the pathogenesis of infectious hepatitis A virus infection and development of immunity in the intestine. To develop methods for enhancement of response to enteric vaccine.

24 (U) Pathogenesis of hepatitis A virus was studied by infecting owl monkeys with this virus. Shortly after the onset of hepatitis, liver biopsy specimens were collected. Tissues were sectioned, stained and examined by light and electron microscopy. Development of IgA immunity in the gut was studied by passive transfer of lymphoid cells between Ig allotype in congenic mice. Lymphoid cells with different membrane characteristics were separated, by rosetting technics, gradient centrigugation, ^{125}IUDR and ^{51}Cr labeling, ^{3}H-thymidne suicide and UV-BUDR killing. Repopulation, migration and lodging were then examined by fluoresent microscopy and isotope counting.

25 (U) 8310-8409 Owl monkeys infected with hepatitis A virus consistently showed hepatocyte destruction although virus particles were not seen. Electron microscopic examination revealed characteristic changes in the livers of infected monkeys. In the study of IgA immunity in the gut, IgA precursor cells in Peyer's patches were characterized as resting IgM-IgD double bearing cells carrying complement receptors on their surface. These cells migrate to mesenteric lymph nodes, through the thoracic duct and blood circulation, reach the spleen, and eventually arrive in the gut lamina propria. During the migration and recirculation these cells asynchronously differentiate into IgA blasts, and plasma cells and lodge in the gut lamina propria. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83 - 30 Sept 84.

DD FORM 1498 1 MAR 83
EDITION OF MAR 68 IS OBSOLETE

PROJECT 3M61102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 222: Histopathologic Manifestation of Military Disease and Injuries

Investigators:
    Principle: Jeenan Tseng, Ph.D
    Associate: Ludmila Asher, MD
    Assistant: SP5 Michael Williams
               SP5 Mildred Lopez

Objective:

Enteric diseases are important medical problems constantly affecting Army personnels. To develop methods for enhancement of the response to enteric vaccines. To determine the pathogenesis of infectious hepatitis virus and cellular development of immunity in the gut.

Progress:

I. Pathogenesis of Hepatitis A Virus:

Although hepatitis caused by infectious hepatitis A virus has been known for years. The pathogenesis of the infection is still obscure. In collaboration with Dept. of Viral Diseases using owl monkeys infected with hepatitis A virus. We have consistently seen mild to moderate destruction of hepatocytes in liver biopsy specimens taken shortly after the peak time of hepatitis. In the limited number of specimens examined, virus particles thus far have not been seen. An immunoperxidase staining technique for localizing hepatitis A antigen was developed. Further studies are required.

II. Cellular Development of IgA Immunity in the Gut:

Humoral immunity of the gut is essentially manifested by IgA antibodies which are produced by plasma cells in the gut lamina propria. Previously, we found that the IgA plasma cells in the gut are derived from precursor cells in Peyer°s patches. These precursor cells migrate to mesenteric lymph nodes, go through the thoracic duct and blood circulation, reach the spleen and eventually arrive in the gut lamina propria where they become resident IgA plasma cells. The efforts were then extended to studying characteristics and migration of the IgA precursor cells. It was found that IgA precursor cells in Peyer°s Patches are mainly IgM-IgD double-bearing cells, they are resting lymphocytes bearing complement receptors. When they migrate to mesenteric lymph nodes and thoracic duct, some of them differentiate into IgA blasts and IgA bearing cells. The IgA blasts migrate directly to and reside permanently in the gut lamia propria and other mucosal tissues. The IgA bearing cells migrate directly and/or indirectly to the gut lamina propria, differentiate after interacting with antigens into IgA blast and plasma cells, and lodge there for the rest of their life. If no interaction with antigens occur, the IgA bearing cells migrate out of the gut lamina propria, reach mesenteric lymph nodes, and migrate and differentiate in a second homing journey. The IgM-IgD double-bearing cells arriving in the gut lamina propria may differentiate into IgA bearing cells, IgA blasts and plasma cells after interacting with antigens, or may migrate out of the gut lamina propria with all the cellular characteristics unchanged, recirculate in the gut tissues, home to the gut lamina propria, and differentiate. This asynchronized cycle of differentiation-lodging-recirculation is probably the theme occurring in the body that results in an IgA preponderance in the gut.

Future Studies:

I. Infection of hepatitis A virus, the tissue site(s) of entry, route of transmission, site(s) of virus proliferation and inactivation will be delineated. Emphasis will be focused on subcellular localization of hepatitis A virus at various stages of infection.

II. Studies of the cellular mechanism and antigenic stimulation that control and affect the migration and lodging of IgA precursor cells and other lymphoid cells are unknown and now being undertaken. The results should provide valuable information for better understanding of gut immunology and proving basic information regarding induction of immunity by oral vaccines.

Publications

1. Tseng, J., Repopulation of IgA plasma cells in the gut lamina propria with lymphoid cells isolated from gut lamina propria. Eur. J. Immunol. 14, 420 (1984).

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA OB 6537	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AI	223 WKPS
b. CONTRACTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Pathologic Manifestations of Diseases of Military Importance

12 SUBJECT AREAS
[illegible] Biology 0613 Microbiology

13 START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16 PERFORMANCE METHOD
74 02	Cont	DA	C. In-House

17 CONTRACT GRANT
a. DATE EFFECTIVE EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE d. AMOUNT
e. KIND OF AWARD f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	8.0	354
85	8.0	351

19. RESPONSIBLE DOD ORGANIZATION
a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
Top, F H Jr
d. TELEPHONE NUMBER (include area code)
202-576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed ARmy Institute of Research
Division of Pathology
b. ADDRESS
Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
Elwell, M R
d. TELEPHONE NUMBER (include area code)
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f. NAME OF ASSOCIATE INVESTIGATOR (if available)
Keenan, K P
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
Andersen, G L

21 GENERAL USE
FINA
MILITARY CIVILIAN APPLICATION H

22 KEYWORDS (Precede EACH with Security Classification Code) (U) Pathogenesis; (U) Morphologic Pathology; (U) RAM (U) Lab Animals; (U) Schistosmiasis; (U) Leishmaniasis; (U) Trypanosomiasis (U) Mice

23 TECHNICAL OBJECTIVE 24 APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23(U) To study and define the pathology and pathogenesis of experimental trypanosomiasis and leishmaniasis and the effects of other infectious, toxic, and environmental bio-hazards in a variety of animal hosts. Initiate and provide pathologic studies needed to prevent/control disease and conditions that impact on quality assurance of the WRAIR-reared and purchased laboratory animals. Provide diagnostic pathology for animals acquiring natural diseases and deaths during quarantine or colonization at the WRAIR. Provide clinical pathology and histopathology support to the WRAIR and other eligible government agencies. All projects are generated from approved protocols and are related to military medical problems.

24(U) Studies utilized conventional gross and histopathology, clinical pathology, histochemistry, immunohistochemistry, and electron microscopy techniques.

25(U) 83 10 - 84 09 A model for the study of visceral leishmaniasis in the aotus monkey was developed using the promastigote form of the organism for the experimental infection. A study was completed on acute renal failure in the rat model. Based upon pathological observations, salicylanilide (WR46243) was an effective, non toxic, antipenetrant for preventing shistosomiasis infection in rhesus monkeys. There was no definative evidence of an adverse systemic effect of chronic implantation of ceramic teeth in baboons. A dengue-2 candidate vaccine was found to have very little tendency for neurovirulence following direct injection into the brain and spinal cord of rhesus monkeys. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

Project 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 223 Pathologic Manifestations of Diseases of Military Importance

Investigators: Principal:	James B. Moe, DVM, Ph.D., LTC(P), VC Gary L. Andersen, DVM, MS., MAJ, VC Michael R. Elwell, DVM., Ph.D., MAJ, VC
Associate:	LuAnn McKinney, DVM., MAJ, VC Richard E. Long, DVM., CPT(P), VC Charles B. Clifford, DVM, CPT(P), VC Robert E. Hunt, DVM., CPT(P), VC Isaac J. Hayward, DVM., CPT, VC Larry W. Mitcheltree, DVM., CPT, VC Mary Ball, DVM., CPT, VC

Description:

To diagnose, define, investigate and compare known and potential diseases common to man and animal, particularly those of military significance. To devise and evaluate means for precise diagnosis, control and/or prevention of inflammation and tissue injury induced by these diseases. To develop new animal models for the study of human diseases. A major effort has been directed toward defining the pathogenesis and fundamental mechanistic events operative at the cellular and subcellular levels during the induction of tissue injury. Studies have applied methods of microscopic pathology, histopathology, clinical pathology, ultrastructural pathology, histochemistry, and immunohistochemistry.

Progress:

Progress in original and collaborative studies is presented below:

I. Studies on Shigella dysenteriae I enterotoxin on the morphology of the rabbit ileum.

Collaborative study with the Dept. Bacterial Diseases, Division of Communicable Disease and Immunology. The effects of crude and purified Shigella dysenteriae I enterotoxin at different doses on the morphology of the intestinal mucosa in the rabbit ileal loop models are being studied. Light microscopy and histochemistry studies are ongoing and transmission and scanning electron microscopy studies have been initiated. Present findings indicate a dose dependent response of the ileal mucosa to both crude and purified enterotoxin preparations. Initial results suggest that direct cytotoxic damage to the absorptive epithelial cells covering the intestinal villus is the first event in the pathogenesis of the enterotoxin action. This results in sloughing of the absorptive epithelium, microulceration, reduction of the villus length (villus atrophy) and reduction of the absorptive surface area. In contrast to the villus epithelium, the undifferentiated crypt epithelium becomes extremely hyperplastic and this cell population rapidly expands to repair and rebuild the atrophic villi. The initial lesions of villus atrophy and crypt hyperplasia suggest a functional deficit leading to malabsorption. However, since the crypt enterocytes have secretory as well as proliferative capabilities, it appears likely that the overall mechanism of diarrhea and fluid production is the result of a net secretion from the hyperplastic crypts overcomming the absorptive ability of the atrophic villi. Ultrastructural studies confirm the above observations. The epithelium is more rapidly lost from the villus and exhibits sublethal changes as early as two hours after exposure to the enterotoxin. The main subcellular change observed is autophagocytosis with digestion of cell organelles within cytoplasmic vacuoles. This suggests that ultrastructural organelles are the target of the enterotoxin. Studies are underway to identify the target(s) and describe the ultrastructural sequence of all damage. Experiments have been completed and tissues fixed and prepared for ultrastructural study.

II. Pathology in C57BL/jb Mice Infected with *Trypanosoma rhodesiense*.

Collaborative study with the Department of Immunology, WRAIR. There is a need for a practical laboratory animal model for the study of chronic trypanosomiasis. The infection of *Trypanosoma rhodesiense* in laboratory rats and mice generally leads to an acute fatal disease without the development of the chronic disease or cerebral lesions so much a feature of the disease in man. In recent years there have been some reports of chronic trypanosomiasis with cerebral lesions in mice infected with *T. equiperdum* and *T. brucei*. However, a rodent model for the study of chronic trypanosomiasis caused by *T. rhodesiense* in still lacking. Initial studies in our laboratories indicated that a chronic infection of trypanosomiasis with significant cerebral lesions could be produced in C57BL/jb mice infected with a human strain of *T. rhodesiense*. These findings led to a more detailed study. One hundred and nineteen (119) mice were inoculated intraperitoneally (IP) with 10^3 *T. rhodesiense* strain ZVH 18A9. The mice were sequentially killed every two weeks PI with the last surviving infected mice killed at 147 days PI. The infected mice became anemic, hypoglycemic, hypergammaglobulinemic (with IgM greatly increased) and hypoalbuminemic. Immunofluorescence studies of the kidneys suggested an immune-complex glomerulonephritis. Lesions histologically similar to those described in chronic trypanosomiasis in man were found in the brain, spleen, lymph nodes, liver, heart, kidney, pancreas, and epididymis. Data on parasitology, immunology and pathology are being compiled. An additional forty (40) mice have been studied by light and electron microscopy to determine additional details of the morphology of the changes in the kidneys and brain. Changes in the kidney, particularly the glomerulus, do not persist and evidence of immune-complex glomerulonephritis was observed in chronically infected mice. However, progressive changes were observed in the brains of C57BL/Jb mice chronically infected with T. rhodesiense. The changes are most evident in the choroid plexus and periventricular cerebral cortex. Extensive inflammatory cell infiltrates were seen in the brains of all chronically infected mice. The ultrastructural details of these changes are presently being documented.

The morphological, hematological, serological and immunological results plus the duration of the infection indicates that the C57BL/Jb mouse infected with this strain of T. rhodesiense makes an excellent model for the study of chronic cerebral trypanosomiasis.

III. Turmorigenesis of N-Butyl Cyanoacrylate in Rats.

Collaborative study with the Division of Surgery, WRAIR. N-butyly cyanoacrylate, a tissue adhesive, was injected subcutaneously in 149 Sprague Dawley rats to determine the tumorigenesis of this agent. This summary describes the gross and microscopic findings in the study. The experimental design consisted of four groups: Groups 1 and 2 received .1ml and .4ml of N-butyly cyanoacrylate, respectively; Groups 3 and 4 (controls) received .1ml and .4ml normal saline respectively. All injections were given in the dorsal subcutaneous tissue between the shoulders. A total of 15 rats were discarded due to severe postmortem autolysis, none of these had grossly visible tumors. Tissues from 134 rats were saved for histology. There were 38 rats in group 1, 35 rats in group 2, 32 rats in group 3, and 27 rats in group 4. Tissues were saved on 2 rats without knowledge of their treatment group. (Tumors were not seen in these rats). A total of 73 rats received N-Butyl Cyanoacrylate of which 18 developed neoplasms consistent with malignant fibrous histiocytoma (MFH) at the subcutaneous injection site. One of these (treated with .1ml N-Butyl Cyanoacrylate) had metastatic neoplastic (MFH) foci in the lung, kidney, heart and adrenal gland. None of the rats receiving normal saline developed MFH at the injection site.

IV. Acute Renal Failure: The renal effects of the intravenous administration of myoglobin and the inhibition of prostaglandin synthesis during hemorrhagic hypotension in rat.

Collaborative study with the Department of Nephrology, WRAIR. Post traumatic renal insufficiency associated with myoglobinuria is a common complication of traumatic injuries. Such injuries expected in the military might include gunshot or shrapnel wounds, crushing injuries, exertional rhabdomyolysis, hyperthermia and misuse of tourniquets. Although many factors have been proposed as the primary factor responsible for renal injury, renal ischemia, heme pigments (myoglobin, hemoglobin), acid urine, and prostaglandin inhibition may contribute to renal failure.

The objective of this experiment was to develop a reproducible myoglobinuric model of acute renal failure in moderately dehydrated, aciduric, and prostaglandin-inhibited rats by the intravenous infusion of purified equine myoglobin during a period of hemorrhagic hypotension. The test animals were 300-350 gram (body weight), male, Sprague-Dawley rats which were divided in 8 groups, one of which was a control group. The other groups, consisted of an indomethacin group, indomethacin plus hemorrhage group, hemorrhage group, myoglobin group, myoglobin plus indomethacin group, myoglobin plus hemorrhage, and myoglobin plus hemorrhage and indomethacin. Experimental animal which recieved more than one test substance received the substances in the following sequence: 1) indomethacin was administered over a 10-15 minute time span; 2) followed 35 minutes later by a period of hemorrhage in which 25% of the rat's estimated blood volume (based upon percent body weight) was removed. 3) Myoglobin was then administered at a mean dose of 600 mg/kg B.W. intraarterially via cannula in the tail artery. Rats were observed for 48 hours following administration of the test substances, during which time blood and urine samples were collected for evaluation. After 48 hours the rats were anesthetized, the kidneys perfused with neutral buffered formalin via the aorta, then the animal was sacrified by exsanguination while anesthetized. Duplicate sections of each kidney were examined histologically. Kidneys were evaluated by 16 parameters and graded subjectively by estimatinng the percent of cortical and medullary tubules affected. In this study, significant differences from the control group were noted in all groups in which myoglobin was administered. There were no significant differences between the control group and the groups which did not receive myoglobin. Myoglobin alone was toxic to the epithelial cells lining the cortical and medullary tubules. Indomethacin possibly contributed to the severity of the inner medullary casts. The lesions seen represent transient, functional, and reversible changes which do not involve enough of the kidney to be considered life threatening. Tremendous variation in the severity of tubular casts was seen within a group, as well as between different groups. These findings suggest that the various models investigated in this study will have limited utility as prototypes for the study of life-threatening renal failure.

V. Experimental Visceral Leishmaniasis in Non Human Primates: Pathologic and Serologic Characterization of *Aotus trivirgatus* infected with *L. donovani* Promastigotes.

Collaborative study with the Department of Immunology, WRAIR. The development of a suitable vaccine for the prevention of visceral leishmaniasis (Kala Azar) would be greatly aided by the availability of a primate model of the disease. A model of visceral leishmaniasis using the owl Monkey (*Aotus trivirgatus*) has been recently developed. Intravenous infection with *L. donovani* amastigotes (32.5 x 10^6 organisms/kg) resulted in severe clinical illness with heavy parasite burdens in the liver, spleen, and bone marrow at 8-10 weeks.

This protocol utilizes this model to determine whether intravenous infection with the 25 strain *L. donovani* (*Sudan strain*) promastigotes results in a similar illness. The promastigote form of the parasite is required for infection in a model vaccine system because: 1. This is the stage which is introduced into the host by infected sandflies and is the stage against which one would ideally direct an immune response. 2. Viable promastigotes can more precisely quantitated than amastigotes thus enhancing the reproducibility of the infectious inoculum. 3. More importantly, BALB/c mice immunized with irradiated *L. tropica* promastigotes, but not amastigotes, have responded with specific anti-leishmanial antibody isotypes (IgG1. 2a, 3) which confered protection from visceral disease following a subsequent intravenous promastigote challenge. These data suggest that relevant protective antigens are recognized on the surface of promastigotes and can be used to immunize and protect mice. Confimatory studies using an *L. donovani* murine immunization model are underway in our laboratory. This work must then be repeated in non human primates.

The study was performed in two phases. During phase 1, the preinfection period, the animals were studied to determine baseline values for the parameters to be observed, and to exclude intercurrent illness which may affect the outcome of the study. During phase 2, the infection period, the animals were followed prospectively, with all procedures and studies performed on both study and control animals. Clinical data is complete and presently being tabulated and analyzed. Three monkeys were euthanized and necropsied after a progressive illness with weight loss and anemia. These monkeys had granulomatous inflammation in the liver and reticular cell hyperplasia in the bone marrow. spleen, and lymph nodes. Leishmania amastigotes were identfied in sections of the liver spleen, bone marrow and lymph nodes of infected monkeys. The remaining monkeys will be necropsied and examined in FY5.

VI. The Efficacy of a Salicylanilide (WR46243) as a Topical Antipenetrant Against Schistosoma mansoni Infections in Rhesus Monkeys.

Collaborative study with the Division of Experimental Theraputics, WRAIR. Because no effective vaccine is available, methods for prevention of shistosomiasis are limited to environmental control measures and prevention of exposure to contaminated water. When exposure cannot be avoided, protective clothing and more recently the use of topical chemical agents can prevent the penetration of the skin by the free-swimming larvae (cercariae) of the schistosome.

Prior to exposure with cercariae, 9 rhesus monkeys were treated with a topical antipenetrant (WR46243) and 4 were not treated. All four non-treated infected controls had characteristic schistosomal ova granulomas in the liver and intestinal tract associated with mild lymphoid hyperplasia, histiocytosis, and eosinophil infiltration of mesenteric lymph nodes. One of these also had a small eosinophilic granuloma in the skin of the distal antebrachium.

Of the monkeys treated with WR46243 prior to exposure only one had any lesions interpreted as probably due to schistosomiasis. This animal had an eosinophilic granuloma in the antebrachial skin and a mild multifocal portal eosinophil infiltration in the liver. One other monkey had a lesion interpreted as posssibly due to schistosomiasis. This was a mild antebrachial dermal infiltration of histiocytes and mast cells. In neither animal were schistosoma ova identified and schistosomulae were not observed in any animal. Based on pathologic observations, WR46243 appeared to have been quite efficacious in preventing patent schistosomiasis. No evidence of topical or systemic toxicity was observed in any monkey.

VII. Changes in arterial blood gases and cardiac rhythm due to endotracheal suctioning of sheep with abnormal lung function.

A collaborative project with the Department of Surgery, WRAIR. Critically ill patients with endotracheal tubes require frequent suctioning to maintain airway patency. Suctioning is known to produce reductions in arterial oxygenation predisposposing the patient to inadequate oxygenation of vital organs. Cardiac dysrhythmias and sudden death have also been reported to occur during or after suctioning, and are thought to be due to hypoxemia and/or vagal stimulation. No experimental evidence exists concerning these phenomena.

The development of hypoxemia and/or cardiac dysrhythmias following endotracheal suctioning may be poorly tolerated in critically ill patients, many of whom already have severly compromised cardiovascular and respiratory function. In order to minimize these complications during endotracheal suctioning, the administration of 5-8 hyperinflation breaths with 100% oxygen immediately before and after suctioning has been recommended. No data have been published on the effectiveness of this intervention to minimize hypoxemia and/or cardiac dysrhythmias. In addition, the few studies on endotracheal suctioning which have been published have utilized experimental animals and hymans with normal lung function.

The purpose of this study is to describe the effects of five hyperinflation breaths with 100% oxygen, administered before and after suctioning, on arterial blood gases and cardiac rhythm in sheep with respiratory insufficiency.

Following intrapulmonary administration of an acidic fluid to produce an aspiration pneumonia, hyperinflation-suctioning was performed and blood gases measured. Sheep were then euthanized and lungs were examined grossly and microscopically to confirm the presence of pneumonia and determine the extent of lesion. The study is approximately 50% completed at this time.

VIII. Lymphodepletion in rabbits with a fatal diarrheal disease.

Collaborative study with the Division of Veterinary Medicine, Dept of Animal Resources and the Dept of Experimental Pathology. In a colony of rabbits from WRAIR there has recently been a problem of diarrhea resulting in deaths. A Clostridium sp. has been tentatively identified as the cause of this disease outbreak. Rabbits from this same colony, randomly sampled as part of the disease surveillance programs were found to be lymphodepleted, primarily in the follicles of the gut associated lymphoid tissues. This lymphodepletion was often associated with the fatal diarrheal disease. A draft manuscript of the pathologic changes has been completed. Studies are now in progress to determine the relationship of the lymphodepletion and diarrheal disease.

IX. Effects of Ketamine Hydrochloride on the Hemogram, Serum Enzyme Activity and other Biological Parameters in Cynomolgus Monkeys (Macaca fascicularis).

Collaborative study with the Department of Animal Resources, Division of Veterinary Medicine, WRAIR. Ketamine hydrochloride is a dissociative anesthetic routinely used to immobilize non-human primates. Properties of ketamine hydrochloride include rapid induction of anesthetic effects, analgesia and short duration of action. Given the extensive use of ketamine hydrochloride in non-human primates, it is important to determine the effects, if any, on various biological parameters including hemogram and various serum enzyme activities. This project was undertaken in an attempt to define these effects, if any, in the adult cynomolgus monkey (Macaca fascicularis).

Ten healthy adult cynomolgus monkeys were used to form two groups of five animals each. These animals had not had exposure to ketamine hydrochloride for at least six weeks. On day 1, a fasting venous blood sample was obtained from each animal at 0, 5 and 15 minutes post-capture. Minimal manual restraint was used and each animal continued to be restrained between blood samples. Additional blood samples were obtained from each animal at 6 and 24 hours after the initial sample, again using only manual restraint. On day 8, fasting blood samples were again collected. Five monkeys (Group A) were manually restrained, had blood collected at 0 minutes and were then given 5 mg/kg body wt. ketamine hydrochloride intravenously. These animals continued to be restrained as needed and had blood taken again at 5 and 15 minutes post-injection. Additional samples were obtained at 6 and 24 hours post-injection using manual restraint. The other five monkeys (Group B) were handled and sampled in exactly the same manner but received their ketamine hydrochloride as a 15 mg/kg intramuscular injection. On day 22, the day 8 procedure was duplicated except Group A and B animals were reversed as to the mode of ketamine hydrochloride exposure.

Routine complete blood counts, extensive serum enzyme and electrolyte analysis as well as isoenzyme analysis of CPK and LDH by electrophoresis were performed on all samples. Data is currently being statistically evaluated in preparation for a manuscript to be submitted to a major veterinary journal.

X. Collaborative Research Project with the Department of Rickettsial Disease, WRAIR Tissue Identification of Rickettsia coroni.

Thirty two mice were infected with Rickettsia coroni to evaluate the pathogenesis of the agent and to identify the agent in various body tissues. The mice were divided into four treatment groups consisting of eight mice per group. Each treatment group was euthanized on separate days and sections of brain, kidney, liver, lung and spleen from each mouse were submitted in 10%

buffered formalin for histologic evaluation. The sections were trimmed and stained with both H & E and Giemsa. All tissues (both stains) were reviewed histologically, however, there were no Rickettsia agents seen. Unstained slides were made and given to the investigator for further use.

XI. Collaborative Research Project with the Department of Nephrology, WRAIR. Effect of Hemorrhagic Hypotenssion and Myoglobin Infusion on Renal Function in the Dog.

A total of twelve female mongrel dogs are to be used in this study. Six dogs have been used to date which were divided randomly into two treatment groups. Group 1 received hemorrhagic hypotension plus myoglobin infusion, and group 2 recevied hemorrhagic hypotension with on isovolumetric infusion of normal saline. The dogs were euthanized, perfused with 10% buffered formalin and the kidneys submitted for histopathological evaluation with no indication given as to treatment group assignment. The kidneys were trimmed and stained with H & E. The following variables were utilized to evaluate histologic changes: tubular dilatation, tubular casts, loss of proximal brush borders, tubular epithelial vacuolization, tubular epithelial necrosis, mitosis, inflammation, mineralization, glomerular change and cast location either with the cortex or medulla. Tissue changes were graded from 1 thru 4 respesenting mvere tubular dilatation and casts, one had moderate changes using these variables, two had mild changes and one had very minimal changes. Other variables ranged from minimal to moderate.

There seems to be differences in tissue morphology based on the kidneys reviewed from these six dogs. The differences may represent the treatments received. All tissues will be reviewed when received from the principal investigator. The study is ongoing.

XII. Changes in Serum CK and LD following heart surgery in dogs Collaborative study with the Division of Surgery, WRAIR.

To determine the changes in serum CK and LD following heart surgery, thoracotomies were performed on twenty dogs after peripheral basline blood samples were drawn. Each subject was placed randomly into one of four groups: thoracotomy alone, placement of atrial purse string sutures, atriotomy, and acute myocardial infarction. Blood samples were drawn at selected intervals for 48 hours from each animal. Serum from each sample was analyzed for total CK and LD by automated spectrophotometery. Agarose gel electrophoresis was used to determine the isoenzyme concentrations. Necropsies were performed on each animal to confirm that the desired injury had been created and that no intercurrent diseases were present. Total serum CK became elevated in all four surgical groups postoperatively. Miximal elevations were reached 12 hours after surgery. CK-MB in international units per liter was more useful for distingusihing acute myocardial infarction than was CK-MB when expressed as a percent of the total CK. Animals undergoing thoracotomies, placement of atrial purse string sutures, and atriotomes had ratios of serum LD_1/LD_2 that were 1.00, but they were significantly less than those seen with a perioperative acute myocardial infarction. In situations where the ratio of serum LD_1/LD_2 is questionable for confirming a myocardial infarction, LD_1 expressed as a percent of the serum total LD are important for making this determination. Elevated ratios of LD_1/LD_2 in conjunction with abnormally higher levels of CK-MB in IU/l confirmed that acute myocardial infarction had occurred.

XIII. Chronic tooth implant study in Baboons.

In a collaborative study with the U.S. Army Institute of Dental Research, sections of a variety of tissue representing the various organ systems from eight baboons were study histologically. These baboons had been recepients of long term ceramic tooth implants. There were no control animals included in the sets of tissues submitted for histopathological examination.

A variety of pathologic lesions and tissue changes were observed in these baboons. Essentially all changes recorded were interpreted as representing spontaneous, clinically insignificant disease, or effects of old age in the majority of cases. An interesting finding was the presence of nodular collections of hyperplastic acidophil cells in the anterior portion of the pituitary, a lesion which is not often observed in other species. The significance of this hyperplastic lesion is unknown, however, it is doubtful that this, or any of the other lesions recorded, reflected an adverse effect of chronic implantation of the ceramic teeth. Further special studies of pituitary tissue from these baboons and study of pituitaries from other baboons are underway in order to better resolve the nature and significance of the changes observed.

XIV. Quality Assurance Pathology Evaluation for Extramural Contracts.

The Division of Pathology continued to serve as a source of Pathology expertise for the Institute. The quality assurance performance of pathology support done by extramural vendors was continued, consisting of reviewing pathology findings and tissues from one subchronic study on the toxicity of compound WR 180,409. Review recommendations clarfied questionable findings and improved the value of the study. A neuropathology study was performed for

Dr. Natth of Mahidol University in Thailand. The results of this study facilitated the approval of a vaccine against Dengue Fever developed for World Health Organization use by Dr. Natth. This cooperative effort by the Division staff improved scientific contacts for the WRAIR worldwide.

XV. Clinical Pathology Laboratory, Histopathology Laboratory Support and Necropsy Support.

The clinical pathology laboratory handled approximately 9,740 requests for hematology and 36,351 determinations for serum biochemistries during the reporting period. The histopathology laboratory processed approximately 8,125 paraffin blocks and 15,714 microslides. A total of 1,398 necropsies were performed by the necropsy laboratory. These three laboratories support research protocols at WRAIR and its overseas laboratories and other government agencies as well as providing diagnostic support for the Institute's laboratory animal facilities.

Publications:

1. Clifford, C.B., Moe, J.B., Jaeger, J.J., Hess, J.L.: Gastrointestinal Lesions in Lambs Due to Multiple Low-Level Blast Overpressure Exposure. Military Medicine, 1984, 149: 491-495.

2. Moe, J.B., Jahrling, P.B.,: Fatal arenavirus infection. Comp. Path. Bull., 1984, 16:3.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

DA OG 6751 | 84 10 01 | DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	CG	224 WWP4
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Functional and Structural Bases of Blast-Related Tissue Injuries

12. SUBJECT AREAS
0603 Biology 0621 Weapons Effects

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	Cont	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	71
85	2.0	155

19. RESPONSIBLE DOD ORGANIZATION
a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H JR
d. TELEPHONE NUMBER (include area code) 202-576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research Division of Pathology
b. ADDRESS Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR Moe, J B
d. TELEPHONE NUMBER (include area code) 202-576-2677
f. NAME OF ASSOCIATE INVESTIGATOR (if available) McKinney, L
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Lab animals; (U) Rats; (U) Guinea pigs; (U) Functional Correlation; (U) Exposure Factors; (U) Blast Overpressure; (U) Vascular Permeability; (U) Vascular Ultrastructural; (U) Pathogenesis;

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code) (U) RAM III

23(U) To determine the finite structural and functional bases of the pathologic changes classically associated with blast-related injury to various tissues, especially in the respiratory and gastrointestinal systems. Correlate structural and functional pathologic changes with various levels and amounts of blast exposure, emphasizing dose ranges which are near the environmental exposure associated with crew operator positions of large field artillery weapons. Study effects of repeated blast over time periods covering up to 14 days to determine cumulative effects and resolution dynamics. Map the relative sensitivities of airways and vessels in the respiratory system. Compare the tissues throughout the body. Determine the effects of blast injury on other parameters e.g., susceptibility to infectious diseases of military importance.

24(U) Conventional morphologic techniques including light and electron microscopy will be used. Other procedures will involve use of substances such as carbon particles, ferritin and horseradish peroxidase to determine vascular permeability and clearance functions. Small laboratory rodents, especially rats and guinea pigs will be the predominant laboratory animals used.

25(U) 83-10 - 84-09 Experiments in laboratory rats revealed an amplification of the pulmonary effects of repeated blast exposures when exposures were done on consecutive days. Pulmonary parenchymal lesions tended to be more severe than airway lesions under these conditons. Extensive studies were done on the pathologic effects of blast exposure in the Bradley Fighting Vehicle (M2) and the Armored Personnel Carrier (M113) defeated by various types of high explosive anti-tank (HEAT) rounds. These studies are in-progress, however, preliminary results reveal no significant differences between the M2 and M113 in terms of blast effects. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 October 1983 - 30 September 1984.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

Project 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 224: Functional and Structural Bases of Blast-Related Tissue Injuries

Investigators: LTC(P) James B. Moe, DVM, Ph.D
Principal: CPT Charles B. Clifford, DVM
CPT Douglas D. Sharpnack, DVM
MAJ Luann McKinney, DVM

Description:

As new weapons systems are developed, it is imperative that consideration be given to the potential effects that these may have on the health and performance of the crews operating these systems. Use of mammals exposed to blast overpressure generated by weapons or other blast-generating devices provides a means of estimating the susceptibility of mammalian tissues to blast overpressures at levels approximating those received by operators of weapons systems. More detailed study of tissues so exposed helps to resolve the biological bases of blast-related injuries. Additionally, the various pathologic structural and functional techniques are useful in determining the complex interaction between blast overpressure and other factors in the modern combat environment. Resolution at the cellular and subcellular level of the biodynamic events leading to tissue injury is essential to an accurate approach to the prevention and treatment of blast-related injury.

Progress:

To determine the finite structural and functional bases of the pathologic changes caused by blast-related injury in the various tissues. Of special interest are injuries which result from exposures similar to those received by artillery weapons crews in the field environment. Structural and functional changes are correlated with various amounts of blast overpressure, emphasizing dose ranges which are near the environmental exposure associated with crew operator positions of large field artillery weapons. Effects of repeated blasts over time periods covering up to 14 days are studied to determine cumulative damage and resolution dynamics. The relative sensitivities of airways and blood vessels in the respiratory system are mapped. The fragility of the pulmonary vascular bed is compared with that of blood vessels in other organs and tissues of the body. Other functional parameters are investigated.

and electron microscopy, as well as special procedures which determine vascular permeability, mucociliary clearance and other functional parameters, are used. Complex procedures designed to determine the effects of blast overpressure exposure on susceptibility to infectious agents will be adapted as the studies progress.

Follow-on studies investigating the effects of repeated blast exposures on the respiratory tract were conducted in collaboration with the scientific staff of the Blast Biology Facility, Lovelace Foundation, Albuquerque, New Mexico. Groups of rats were exposed to 20 repititions of blast at 1 minute intervals at peak overpressures of approximately 25 pounds per square inch (psi). Studies were conducted at various intervals (1-5 days) after exposure to this regimen on one day or on two consecutive days. The rats exposed on only one day had lesions comparable to those seen in previous studies, i.e., mild pulmonary parenchymal changes and loss of tracheal and bronchial lining cells. Exposure to blast on consecutive days resulted in amplification of the pulmonary parenchymal lesions, expressed mainly as pulmonary hemorrhage. This sequential study revealed the early stages of resolution of the pulmonary parenchymal injury resulting from blast exposure. On post exposure days 1 and 2 alveoli were flooded with blood. By days 3 and 4, there was a mild infiltration of macrophages and formation of numerous hemoglobin crystals. By 5 days after exposure, significant foci of fibrosis were evident in the pulmonary parenchymal. Because this latter finding had been unexpected, studies at later intervals were not carried out. There are, however, serious implications for the potential significance of pulmonary fibrosis for military personnel exposed to either operator-level or casualty-level blast. Further studies are planned.

A comprehensive study on the effects of exposure to blast behind aluminum armor was carried out in collaboration with the Ballistics Research Laboratory, the US Army Aeromedical Research Laboratory, and the Divisions of Surgery and Medicine, Walter Reed Army Institute of Research. This study was designed to determine the relative biological effects of blast overpressure developed in the Bradley Fighting Vehicle (M2) and the Armored Personnel Carrier (M113) when defeated by various types of high explosive antitank (HEAT) weapons (RPG-7 tow, 456). Sheep and pigs were used as the test species, approximately equal numbers of each species of animal being placed in various crew positions in each vehicle. Anesthetized animals were placed in such a manner as to avoid the path of the HEAT-generated plasma jet through the vehicle and the range of the spall cone generated by the penetration, i.e. all effects would be referable to either blast overpressure or noxious gases. Euthanasia and necropsy were performed between one and five ours following exposure. All gross lesions were documented by photography and recorded using the WRAIR Standardized Blast.

Comparison of the incidences annd severities of grossly - detectable tissue changes failed to reveal any clear cut differences between animals in the M2 and M113 vehicles defeated by various types of HEAT rounds (Tables 1 & 2). Because there were no discernible differences between sheep and pigs in any exposure group or between the different angles of fire, pathology data were grouped by vehicle and threat with no further subdivision.

Grossly-detectable blast-type time injuries were observed in the larynx of one animal in the M113 with the RPG-7 threat and in one animal in the M113 with the M456 threat. The same animal in the M113 with the RPG-7 threat also had grossly detectable blast-type injury in the trachea. These laryngeal and tracheal injuries were of moderate and marked severity, respectively.

Tissue changes in the lung which were interpreted on a gross basis as consistent with blast-injury were recorded in only three animals: One in the M-2 (RPG-7 threat) and two in the M113 (RPG-7 threat and TOW threat). All were of minimal or mild severity.

With the M456 threat two of the 4 animals in the M2 had minimal blast-type injuries in the small intestine. One of 4 animals in the M113 with the M456 threat had a mild blast-type injury in the large intestine. In other exposure groups, blast-type injury occurred in the stomach or intestines in a small proportion of animals and was minimal or mild in all cases.

Singeing of the hair or wool occurred in several of the animals in both vehicles with the TOW and 456 threats. There was, however, no hyperemia or blistering of the underlying skin, indicating that there was no thermal injury of the skin.

Skin wounds which contained metal fragments or which had a definite entrance and exit wound were interpreted as shrapnel wounds. Such wounds were identified in 4 animals: 2 animals in the M2 with the RPG-7 threat and 2 in the M113 with the TOW threat. These metal frgments were generally small, less than 1.0 cm in greatest dimension and less than 5.0 grams in weight. None of the fragments had a soft tissue path length of more than 1 cm.

Emphasis is placed on the fact that these data for incidences of blast-type tissue injury are based on gross observations only and do not represent conclusive or final findings. Microscopic examination of tissues, now in progress, will provide definitive information regarding type and incidence of tissue changes in the various experimental groups of animals and will be included in a final report.

Publications

1. Clifford, C.B., Moe, J.B., Jaeger, J.J., and Hess, J.L. Gastrointestinal Lesions in Lambs due to multiple low-level overpressure exposure. Mil. Med. 149: 491-495, 1984.

TABLE 1: GROSSLY DETECTABLE TISSUE CHANGES IN ANIMALS EXPOSED TO THE INTERNAL ENVIRONMENT OF THE M2 AND M113 ARMORED VEHICLES HEAT ROUNDS

Type of Weapon	456		CONTROL (NO FIRING)	
Type Vehicle	M2	M113	M2	M113
No. animals examined	4	4	7	8
Larynx Blast type change (Subjective Severity Score Score-SSS)	0	.25[1](3.0)[2]	0	0
Trachea Blast type change (S.S.S.	0	0	0	0
Turbinate Blast type change (S.S.S.)	0	0	0	0
Lung Blast type change (S.S.S.)	0	0	0	0
Stomach Blast type change (S.S.S.)	0	0	0	0
Small Intestine Blast type change (S.S.S)	.50(1.0)	0	0	0
Large Intestine Blast type change (S.S.S.)	0	.25(2.0)	0	0
Skin Thermal Injury	0	0	0	0
Shrapnel wound	0	0	0	0

1. Incidence of tissue changes expressed as a decimal reflecting proportion of the total animals examined in each group. Data for pigs and sheep, including all angles of fire (combined in each grouping).

2. See Footnote Table 2.

TABLE 2:

Type of Weapon	RPG-7		TOW	
Type of Vehicle	M2	M113	M2	M113
No. animals examined	18	18	13	18
Larynx Blast type change (Subjective Severity Score-SSS)	0	.06[1](4.0)[2]	0	0
Trachea Blast type change (S.S.S.)	0	.06(4.0)	0	0
Turbinate Blast type change (S.S.S.)	0	0	0	0
Lung Blast type change (S.S.S)	0.06(1.0)	.06(1.0)	0	.05(2.0)
Stomach Blast type change (S.S.S)	.06(2.0)	0	0	0
Small Intestine Blast type change (S.S.S.)	0	0	.08(1.0)	.05(2.0)
Large Intestine Blast type change (S.S.S.)	0	0	0	0
Skin Thermal Injury	0	0	0	0
Shrapnel Wound	.11	0	0	.10

1. Parenthetic figures reflect the mean subject severity score (S.S.S.) in the animals which were affected by the tissue change 1 = minimal, 2 = mild, 3 = moderate, 4 = marked (Score adjusted for background). Minimal is the smallest or least extensive lesion detectable grossly, marked is the greatest possible lesion of a given type, mild and moderate are intermediate severities reflecting progressively greater involvement, relatively.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OG 6767	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636	

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	BC	225 WWL3
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/4			

11. TITLE (Precede with Security Classification Code)
(U) Pathophysiology of Blast Injury

12. SUBJECT AREAS
0619 Stress Physiology 0621 Weapons Effects

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	288
85	2.0	386

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Division of Surgery Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR GRAEBER, G M
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-3791
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) PAUSTIAN, P W
	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Blast injury; (U) Tissue markers; (U) Serum markers; (U) CPK; (U) LDH; (U) Isoenzymes; (U) Alkaline phosphatase (U) RAM IL

23 TECHNICAL OBJECTIVE 24 APPROACH 25 PROGRESS (Precede text of each with Security Classification Code)

23 (U) Recent work from this laboratory has shown that serum enzyme systems (particularly CPK) change with bowel infarction. In order to assess properly the changes in various enzyme systems in the peripheral serum subsequent to blast injury, more must be known concerning each enzyme's distribution in the various parts of the GI tract, lung, heart, and in other organs. If a difference in enzyme distribution could be detected, then earlier stages of injury may be detected by assaying the changes in the isoenzymes in the peripheral serum after blast injury. There is military relevance in this research.

24 (U) Our program of serum analyses is being integrated into the program currently being conducted in conjunction with the Department of Clinical Physiology of the Division of Medicine, WRAIR. The enzyme levels in the gastrointestinal tissues, heart, lung, and other tissues is being assayed.

8310 - 8409

25 (U) Work was completed which showed that alkaline phosphate is a less sensitive serum marker of small bowel injury than CPK. Further work is being published which delineates the distribution of CPK isoenzymes in the GI tract from the esophages to the colon. This work described the concentrations of CPK in the mucosa as well as the muscularis. A third study which showed that the isoenzymes of CPK are similar in atrial and ventricular myocardium while the distribution of LDH isoenzymes differs between the two has been published. A fourth work which measured serum levels of CPK, LDH, and lactate in correlation with various levels of pulmonary injury suffered with varying degrees of blast exposure was also completed. This showed that none of these serum parameters changed significantly in the early, immediate post-exposure period (90 minutes after injury). For technical report see WRAIR Annual Progress Report 1 Oct 83 - 30 Sep 84.

DD FORM 1498 83 MAR   EDITION OF MAR 68 IS OBSOLETE.

Project 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 225 Pathophysiology of Blast Injury

Principal Investigator:
LTC Geoffrey M. Graeber, MC

Background and Objectives:

Exposure to certain levels of blast has been shown to cause injuries to specific organs. Certain frequencies and duration of blast will cause injury to the lungs and to portions of the gastrointestinal tract, especially the stomach, the colon, and, to some degree, the small bowel. Our work has been directed at delineating peripheral serum markers, particularly enzymes, which can be used to detect injury to the gastrointestinal tract and to the lungs. Our efforts to date have centered mainly on the gastrointestinal tract. Our early work focused on severe ischemic injury to the small bowel.[1-2] This showed that creatine phosphokinase (CPK), rises in the peripheral serum within 6 to 9 hours after severe injury to the arterial supply to the bowel. Each of the three isoenzymes of CPK rise within the serum in a characteristic pattern: CPK-BB rises earliest and diminishes back to its normal level by 24 hours. CPK-MM rises to a peak value between 12 and 18 hours after injury then diminishes back towards normal limits in the ensuing 24 hours. This isoenzyme has the greatest serum concentration of any of the isoenzymes of CPK after mesenteric infarction. CPK-MB rises in the serum also but tends to rise later and tends to be a marker of more severe injury.[1,2] Similar changes occurred in peripheral serum CPK after infarction of the colon.[3] With such severe injury, CPK is the most reliable indicator of the critical nature with lactate dehydrogenase (LDH) being somewhat less helpful in that it does not rise to as great an extent and its isoenyzmes do not change with a reliable pattern.[3] These changes in serum CPK may also be seen in experimental strangulated obstruction.[4] Hence, if a severe injury were to occur somewhere in the gastrointestinal tract and were to be sequestered somewhere in the peritoneal cavity, the injury would still be able, theoretically, to cause changes in peripheral serum CPK. Similar findings have been recorded for patients both in our laboratory[5] and from other institutions.[6]

There has been some interest in alkaline phosphatase as a marker of severe bowel injury. Experimental results from our laboratory suggest that alkaline phosphatase does rise after severe mesenteric infarction but that it does not rise as fast as CPK nor does it produce levels as high as CPK in the peripheral serum.[7] Since its isoenyzmes do not change appreciably and since its isoenzymes are found in an irregular distribution through tissue samples taken from various organs, alkaline phosphatase is not regarded as a very good protential serum marker of major injury to the gastrointestial tract.

Work published by us within this last year has shown that CPK is distributed throughout the gastrointestinal tract and has particularly high concentrations in the muscularis.[8] Further work has been conducted to delineated the concentrations of CPK and lactic dehydrogenase in the skeletal muscles of the chest wall in the cardiac chambers. A preliminary report has been published on this material[9] which showed that the chest wall muscles have all three isoenzymes of CPK present but that the majority is CPK-MM. Small quantities of CPK-MB are also present in these muscles. The concentrations of CPK-MB in skeletal muscles could cause problems in delineating injury to the heart and to other organs.[9] The complete work is soon to be published and delineates the exact concentrations of isoenzymes in the sketal muscles with respect to the myocardium of each of the cardiac chambers.[10]

Progress:

Work which delineated the capabilities of alkaline phosphatase to measure severe mesenteric injury was finished. As a result of this work the alkaline phosphatase isoenzyme system was considered an inferior marker of bowel injury to creatine phosphokinase. Work is continuing to delineate a new animal model for mesenteric injury other than the canine. Preliminary results for the swine seem to suggest that this animal will be appropriate. Initial work on the sheep has shown that the sheep is not satisfactory either with respect to CPK or LDH.

The experiments which delineated the distribution CPK throughout the gastrointestial tract from the esophagus to the colon were completed and terminated. This work was also done in the canine model, and other alternative animal models are being evaluated at the current time. Work continues in delineating a new model using a different species which can be used in future blast experiments.

Work was initiated and completed which measured the serum level of a CPK, LDH, and lactate in correlation with various levels of pulmonary injury suffered with different degrees of blast exposure. This was conducted in a sheep model. Delineations of the three parameters were taken over a 90 minute period before exposure and a 90 minute period after exposure. These parameters did not change in the exposed animals when they were compared to paired exercise controls. The period of exposure, however, was relatively short. In our previous studies with severe mesenteric injury, changes in the isoenzyme systems usually occurred starting at three hours after injury and progressed to maximum values sometime between 9 and 18 hours after the insult. Further long range studies are anticipated for this year. These studies hopefully will be able to delineate serum enzyme changes with specific organ injury with longer study periods after blast exposure.

Recommendations For The Future:

Work is being carried out at this time to delineate new animal models for exposure to blast. The sheep does not look very promising at this time, however, the swine appears to have appropriate levels of creatine phosphokinase and lactate dehydrogenase. Further work will be conducted to see if the swine will continue to look as promising and will have enzyme systems which are totally comparable to man and to the canine. Further tests on serum from swine and on tissue samples from various organs should be completed this year.

Further work will be done this year on exposing animals to blast and then measuring their serum parameters over a longer period of time than 90 minutes. This should give us some indication as to what would be occuring over a longer term after blast exposure and should allow some determination as to whether serum proteins, enzymes, and electrolytes will change with longer periods of observation. Our previous work[1-4] suggests that a time period, greater than 90 minutes, is necessary to detect the injuries.

PROJECT 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 225 Pathophysiology of Blast Injury

Literature Cited:

References:

1. Graeber, G.M., Cafferty, P.J., Reardon, M.J., et al.: Elevations of Serum Creatine Phosphokinase (CPK) in Experimental Mesenteric Infarction. Surg Forum 31: 148-150, 1980.

2. Graeber, G.M., Cafferty, P.J., Reardon, M.J., et al.: Changes in Serum Total Creatine Phosphokinase and Its Isoenzymes Caused by Experimental Ligation of the Superior Mesenteric Artery. Ann Surg. 193: 499-505, 1981.

3. Graeber, G.M., Wukich, D.K., Cafferty, P.J., et al.: Changes in Peripheral Serum Creatine Phosphokinase (CPK) and Lactic Dehydrogenase (LDH) in Acute Experimental Colonic Infarction. Ann Surg 194: 708-715, 1981.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA OG 6768	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY	61102A	3M161102BS10	BC	226	WWL2
b. CONTRIBUTING					
c. XXXXXXXXXX	STOG 82/83-6.2/4				

11. TITLE (Precede with Security Classification Code)
(U) Pathophysiologic Studies of Blast Injury to the Gastrointestinal Tract

12. SUBJECT AREAS
0619 Stress Physiology 0611 Life Support

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	369
85	2.0	535

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Division of Surgery Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR HARMON, J W
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-3791
21. GENERAL USE FIMA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) BASS, R g. NAME OF ASSOCIATE INVESTIGATOR (if available) AMRAN C KINNEY, R

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Blast injury; (U) Gastrointestinal hemorrhage; (U) Gastrointestinal perforation; (U) Combat Casualty Management; (U) Lab animals; (U) Sheep;

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code) (U) RAM II

23(U) Our technical objective will be to study the pathophysiology of the development of mucosal and serosal hemorrhage, which is known to be a consequence of blast injury and which may be an important aspect of combat casualty management in future conflicts. Low level exposure to blast injury is experienced by troops firing weapons. Much higher levels of blast injury is experienced by troops in the vicinity of an explosion, or in a tank which is struck by a projectile. Extremely high levels of blast overpressure would be experienced by troops in the field of a Fuel Air Explosion (FAE) Mine Neutralization System.

24(U) Our approach to studying blast induced gastrointestinal injury is multifaceted. In Albuquerque we carried out a field study to correlate a range of blast with pathologic injury and serum enzyme levels. At WRAIR we are building a laboratory blasting device. In collaboration with the Department of Surgery at Johns Hopkins we are assessing the ability of Xenon imaging to diagnose blast induced gastrointestinal injury.

25(U) 83/10 - 84/09 We found that a dose dependent range of gastrointestinal injury occurred in sheep exposed to HE induced blast waves, and that current laboratory blood tests were non-diagnostic. We found that Xenon has some capacity to diagnose gastrointestinal blast injury. We became operational with our laboratory blasting device at the conclusion of this year and began calibration of our monitoring systems. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83 - 30 Sep 84.

Project: 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit: 226 Pathophysiologic Studies of Blast Injury to the Gastrointestinal Tract

Invesitgators:
Principal: John W. Harmon, LTC, MC
Co-Investigators: Eugene J. Schweitzer, CPT, MC
Amran Cohen, CPT, MC
John Sampson, CPT, MC
Donald Cousar, MD
Jon Jaffin, CPT, MC

Background and Objectives:

With blast overpressure injury, it is common to see gastrointestinal tract injury. The lesions observed acutely range from petechia in mild form progressing to large hematomas in the submucosa and in the most severe cases including perforation of the bowel.[1-7] The hematomas occur most commonly in the stomach and the proximal colon. They also occasionally are seen in the small bowel and retroperitoneum. At present there is no reliable means to diagnose these lesions. In addition the natural history of these lesions is not known. A knowledge of the natural history of the lesions is of importance for those who will be managing Blast Injury Casualties. If the lesions resolve over time, they are not a significant problem. If, however, they progress to perforation, they are a very major problem. At this time surgeons do not have guidelines for managing non-perforating bowel lesions of blast overpessure.

Progress:

During this fiscal year, we carried out two projects in the area of gastrointestinal effects of blast injury.

First we collaborated with Dr. Gregory Bulkley of Johns Hopkins University, Department of Surgery, to evaluate the potential of the Xenon washout technique for diagnosing the intramural hematomas that result from blast injury. Dr. Amran Cohen, a resident on elective from WRAMC, was the lead investigator in this intensive evaluation which lasted three months. Xenon is an inert, lipid soluble, radioactive gas. After it is injected into the peritoneal cavity, it is removed from tissue with a washout curve that depends on blood flow. We evaluated the application of the intraperitoneal ^{133}Xenon washout technique, previously described for the diagnosis of early intestinal

Table 1. Interpretation of Visual Images

	Strangulated Loop	SMA Occlusion	Intrabdominal Hematoma	Retroperitoneal Hematoma
Sensitivity	100% (28/28)	100% (26/26)	75% (15/20)	91% (29/32)
Specificity	79% (23/29)	79% (23/29)	79% (23/29)	79% (23/29)
Predictive Value Positive Reading	82% (28/34)	8% (26/32)	71% (15/21)	82% (29/35)
Predictive Value Negative Reading	100% (23/23)	100% (23/23)	82% (23/28)	88% (23/26)
Efficiency	89% (51/55)	89% (49/55)	77% (38/49)	85% (52/61)

ischemia, to the detection of such hematomas. Hematomas were placed in the abdomen and retroperitoneum of anesthetized rats. The elimination of ^{133}Xenon from the peritoneal cavity was then monitored for 90 minutes after its injection by external gamma counting and gamma camera imaging. The results from these animals were compared with those from control and sham-operated rats, and from rats with global and segmental intestinal ischemia. The elimination of gamma activity from both groups of rats with intraabdominal hematomas was significantly delayed, when compared with that from control or sham-operated rats, but not to the degree seen with either of the ischemia intestinal lesions. "Blinded" reading of the gamma camera images proved moderately sensitive, specific, predictive, and efficient for the detection of a hematoma, but less so than for the ischemic lesions. By both standards, retroperitoneal hematomas produced more delay in xenon washout than did intraperitoneal hematomas, and these rats were hence more recognizable as abnormal. This technique shows promise for use, either alone or in combination with other techniques, for the detection of intraabdominal hematomas. Table 1 summarizes the results of our testing of the Xenon technique.

The other project in this work unit is the building of a laboratory blasting device. This device has now been built at WRAIR. The pathophysiology of blast injury as well as the effects of various treatment regimens could be nicely evaluated if this system works. It is patterned on a similar device that was built at Porton Downs in England, but which has only had initial evaluation there. At this time the device is undergoing preliminary testing. It produces blast waves of about one msec duration with peak pressures of 70-100 psi. These waves are lethal for rats in preliminary tests. A protocol describing our plans for the initial program of experiments with this device is attached as appendix A.

Another project in this work unit was the evaluation of standard laboratory blood tests including the SMA_{12} and the measurement of creatine kinase and lactic dehydrogenase as means of diagnosing blast injury to the alimentary tract. Experiments were carried out in sheep at Lovelace in a field study. A range of injury was observed after short duration blasts of 3, 15 and 60 psi. A full report of the correlation between injury and laboratory parameters is being prepared.

Recommendations for the future:

Having evaluated Xenon washout we see that it has some potential as a means of diagnosing gastrointestinal blast injury. A mansucript on its use is being submitted for publication. The complexity of its use makes it basically unsuitable for forward field use where this diagnosis would ideally be made on the battlefield. Accordingly a first priority with our laboratory blasting device will be to test peritoneal lavage in this regard. Peritoneal lavage does have potential for field use.

Project:	3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS
Work Unit:	226 Pathophysiologic studies of blast injury to the gastrointestinal tract.

References:

1. Adler, J., MD. Underwater Blast Injury. Medical Bulletin of the US Army, Europe 38(7/8):33-35, 1981.
2. Hamit, H.F., MD, MS. Primary Blast Injuries. Industrial Medicine 42(3):14-21, 1973.
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4. Cameron, G.R., Short, R.H.D., and Wakeley, C.P.G. Abdominal injuries due to under-water explosion. The British Journal of Surgery 31(121):51-66, 1942.
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6. Cameron, G.R., Short, R.H.D., and Wakeley, C.P.G. Pathological changes produced in animals by depth charges. The British Journal of Surgery 39(117):49-69, 1941.
7. Harmon, J.W. and Haluszka, M. Care of Blast-Injured Casualties with Gastrointestinal Injuries. Military Medicine 148:586-588, 1983.

APPENDIX A

PROTOCOL (Revised 27 Sept 1984)

TITLE: Blast Effects on the Rat and the Usefulness of Peritoneal Lavage in Diagnosis of Intra-abdominal Blast Injury.

DIVISION: Surgery

DEPARTMENT: Gastro-intestinal Surgery

RESPONSIBLE INVESTIGATORS:

Principal Investigator(s):	John W. Harmon, MD, LTC, MC Dennis Moritz, MD, MAJ, MC Luann McKinney, MAJ,MC Jonathan Jaffin, MD, CPT, MC
Department Chief:	Richard C. Kinney, MD, CPT, MC
Division Director:	John W. Harmon, MD, LTC, MC

COORDINATION:

When protocols require support from other Divisions, investigators must coordinate with the Divisions involved and obtain signatures.

Supporting Division(s): Pathology	James B. Moe, DVM, Phd, LTC(P), VC

MANAGEMENT DATA

Short Title (30 characters: Blast and Peritoneal Lavage

Task: Starting Date: 1 October 1984

APC: WWL3 Ending Date: 30 September 1985

USDA Code: D

Species/Strain/Stock:	Sex	Age/Wt	Total #	Ave#/Ave Days Housed
Sprague-Dawley Rats	M/F	200-300 gm	83	20 - 7 days each

Background:

Conventional high explosives as well as nuclear weapons are capable of producing significant blast overpressures. Blast injuries are generally divided into two categories-direct and indirect. Direct blast injuries are those due solely to the effects of the transient overpressure. Indirect blast injuries are those due to displacement of the victim's body or material driven into contact with the victim's body.

A number of studies of blast injuries were stimulated by WWII. They generally found that the lethal injuries were most frequently pulmonary, but associated abdominal injuries were frequent-61 percent in the studies of Clemedson [1]. Since the 1950's the Lovelace Foundation for Medical Education and Research has been continuously involved in studying the biological effects of overpressure. Their work has generally used large shock tubes to generate the blast wave and has been focused on the pulmonary injuries. [2,3,4] Walter Reed Army Institute of Research has been involved in blast research for a number of years. A recent review of blast generated gastrointestinal injuries noted the relative absence of classic signs and symptoms, and the lack of knowledge of the role of peritoneal lavage in diagnosing these injuries. [5] It is also possible that repeated blasts involving lower overpressure may result in more significant intestinal injuries without fatal pulmonary injury.[6]

In order to more carefully study the effects of blast on small animals, a number of air driven shock tubes or blast chambers have been utilized. A recent blast generator was developed at the Trauma Section of the British Chemical Defense Establishment. This device utilizes compressed nitrogen which bursts an aluminum disc to generate the shock wave. The wave seems highly reproducible, and has a very short lethal range (less than 5 cm), thus permitting its safe use in the laboratory. This device, in association with appropriate blast and physiologic monitors, should allow more detailed investigation of the basic mechanisms of blast injury and possible diagnostic and therapeutic modalities. No reports of results with this particular device have been published. The initial studies will determine the reproducibility of these blasts and resultant physical injury. In addition, the effects of thoracic blast on pulmonary function as measured by arterial blood gases and the usefulness of peritoneal lavage to assess intra-abdominal injuries will be assessed.

Rationale: The rat has been selected as the smallest mammal which can be appropriately instrumented and monitored.

Hypotheses:

1. Reproducible blast injuries can be generated utilizing a nitrogen gas generated shock wave in a small scale apparatus.

2. The injuries sustained by a rat in this device will be similar to these sustained by larger animals in tests involving high explosives on shock tubes..

3. The diagnosis of life threatening pulmonary injury can be assisted by arterial blood gas measurements made shortly after injury.

4. The diagnosis of life threatening abdominal injury can be assisted by peritoneal lavage done shortly after injury.

Objectives:

1. Produce consistent injuries to a rat utilizing a small scale compressed nitrogen gas shock wave generator.
2. Compare these injuries to those expected from shock waves generated by high explosives or shock tubes.
3. Correlate early changes in arterial blood gases after injury with the presence of life-threatening thoracic injuries.
4. Correlate the results of peritoneal lavage done shortly after injury with the presence of life-threatening abdominal injuries.

Materials and Methods:

Materials:

A. The Blast Generator consists of a portable apparatus consisting of a large Compressed Nitrogen storage tank, a 150 cc pressure flask, a solenoid operated valve, a blast nozzle, and appropriate tubing and valves. The pressure flask is charged from the large compressed nitrogen storage bottle to 1500 psig. When the solenoid valve is activated (opened), this pressurized gas is rapidly admitted to the space in the blast nozzle behind the aluminum disc. When the disc ruptures, a blast wave is generated and exits the nozzle ahead of the escaping nitrogen. A device at the exit of the blast nozzle captures the aluminum disc, so no fragments exit the nozzle. A series of test firings has already demonstrated the safety of the device, the fact that no particulate matter exits the nozzle. Furthermore, the blast wave has been generally categorized as a very short duration overpressure of high intensity. The device will be operated in accordance with a Standard Operating Procedure. Hearing protection is required in the immediate vicinity (same room) when testing is in progress. British data (personal communication) confirms that the test animal is only subjected to a blast overpressure with no external injury inflicted. A schematic of the blast generator is included with this protocol.

B. The Test Platform consists of a table for the target animal and mountings for pressure detectors. These pressure detectors will measure blast wave characteristics in microsecond time frames and are mounted both perpendicular and parallel to the blast wave in order to measure the incident and stagnation overpressures respectively. The pressure detectors can be moved in 2 planes with respect to the target animal, and the entire assembly then positoned an appropriate distance from the blast nozzle.

C. The Blast Monitor consists of a Piezotronics Model 43A08 Power Supply for the pressure detectors coupled to a Nicolet Model 4094 Digital Oscilloscope. Blast data will be stored in digital form on diskettes by the Nicolet XF-44 dual disc recorder and displayed as required by a Hewlett-Packard Model 7470A digital plotter.

D. Physiological Monitors will consist of standard Arterial Blood Pressure monitoring equipment (Tektronics, Inc), EKG monitors, and routine Arterial Blood Gas testing.

Method: Test animals will be Sprague-Dawley rats between 200 and 300 grams. The animal will be weighed and then anaesthetized with an intramuscular injection of

Ketamine (44 mg/kg) and Rompun (8.8 mg/kg). Additional IM injections of anesthetic will be administered as needed during the procedure to ensure an adequate level of anaesthesia.

A. The initial experiments will involve generation of blast waves and their characteristics using the blast monitor. No animals will be used. Basically the pressure patterns and durations will be described.

B. The next experiments will describe the injuries produced by the blast including minimal injuries with anticipated full recovery and allow comparisons with other blast models such as high explosive or shock tube. To carefully define the anatomic injury, each group will consist of 5 animals. A group of 5 animals will be exosed to blasts at 4 cm, 3.75 cm, 3.50 cm, 3.25 cm, 3.0 cm until an 80 percent short term (1 hour) mortality is encountered. It is expected that this will occur at about 2.5 cm. Thurs, a total of about 7 groups of 5 animals each will be used.

For these experiments, the anaesthetized animal will be placed on the test platform, the blast geometry will be established, and the blaster triggered. Response of the animal, especially respiratory patterns, will be recorded. Surviving animals will be euthanatized at one hour with an overdose of Intra peritoneal Pentobarbitol (80 mg/kg). These animals will have appropriate necropsies performed by Major McKinney and the thoracic and abdominal injuries graded with a 3 point system for no, mild, severe injury.

C. After completion of the above descriptive phase, a trial of peritoneal lavage as well as blood pressure response to blast effects will be conducted. The test animals will be anaesthetized with an intramuscular injection of ketamine (44 mg/kg) and Rompun (8.8 mg/kg). Additional IM injections of Ketamine and Rompun will be administered if needed during the procedure.

A small incision will be made over the carotid artery and a # 50 PE Tube inserted. This surgical site will then be closed, and the animal moved to the test platform. The blast geometry will then be established, and the blaster triggered. Physiological monitoring will continue after the blast for one hour or longer if the animal is not stable at that point.

At 30 minutes after the blast, the peritoneal lavage will be conducted. A very small midline incision will be made and carried down into the peritoneal cavity. A small catheter will be inserted and held in place with a pursestring suture. About 10 cc of sterile Lactated Ringers will be introduced into the abdomen, and then removed. This fluid will be examined for gross or microscopic evidence of blood or fecal material, as well as amylase level. The White Blood Cell Count and serum amylase will be measured at this time.

At 60 minutes after the blast an exploratory laparotomy will be conducted via a midline incision. Evidence of damage to include perforation, bleeding, subserosal or intramural hematomas of the bowel wall will be sought.

After the exploratory laparotomy, all wounds will be closed and catheters removed. The animal will be allowed to recover.

Autopsies will be performed on animals that are fatally injured. Surviving animals will be euthanatized 7 days after injury using an overdose of intraperitoneal pentobarbitol (80 mg/kg) and autopsied at that time.

Groups of 6 animals will be tested at each appropriate range. The first group will be tested at the range found to cause a 50 percent mortality in Section 6. Tests will then be set at 0.25 cm intervals until no pathological changes are found. This is expected to be about 1.0 cm beyond the range for 50 percent mortality. Additionally, three control groups are needed: one with blast only(no peritoneal lavage or laparotomy); one with only peritoneal lavage (no blast or laparotomy); and one with peritoneal lavage and laparotomy (no blast). These should determine if any damage found at autopsy was due to the peritoneal lavage or laparotomy. Thus a total of 8 groups of 6 animals are needed.

EXPERIMENTAL GROUP.

A. Descriptive Phase - 7 groups of 5 to reach 80% mortality.

B. Peritoneal Lavage and blood pressure response - 8 groups of 6 as described above.

Total animals required - 83.

Data Analysis:

A data sheet similar to that attached will be prepared for each animal. The hypothesis that these injuries will be similar to those seen in shock tube or explosive trials will be evaluated in a subjective and descriptive manner. The hypotheses regarding reproducibility of injury, correlation of changes in blood gases with thoracic injury, and correlation of peritoneal lavage with abdominal injury will be tested using analysis of variance.

Blast Injury Protocol:

Date:______ Sequence Number: _____

Animal Tail Mark: _______ Weight:______

Blast: Distance _________cm; Centered: Chest-CM, Abd.

Pressure, Incident ____ psig, Stagnation______psig

Recorded - Disc No.______ Record No.______

Apneic time ______ loss of BP time _____

Time (min)	PO2	PCO2	Ph	Notes

Peritoneal lavage:

Gross: (blood, material): ___________

Microscopic: RBC/hpf & material: ______

Amylase - fluid Serum Amylase _______

CBC ________________

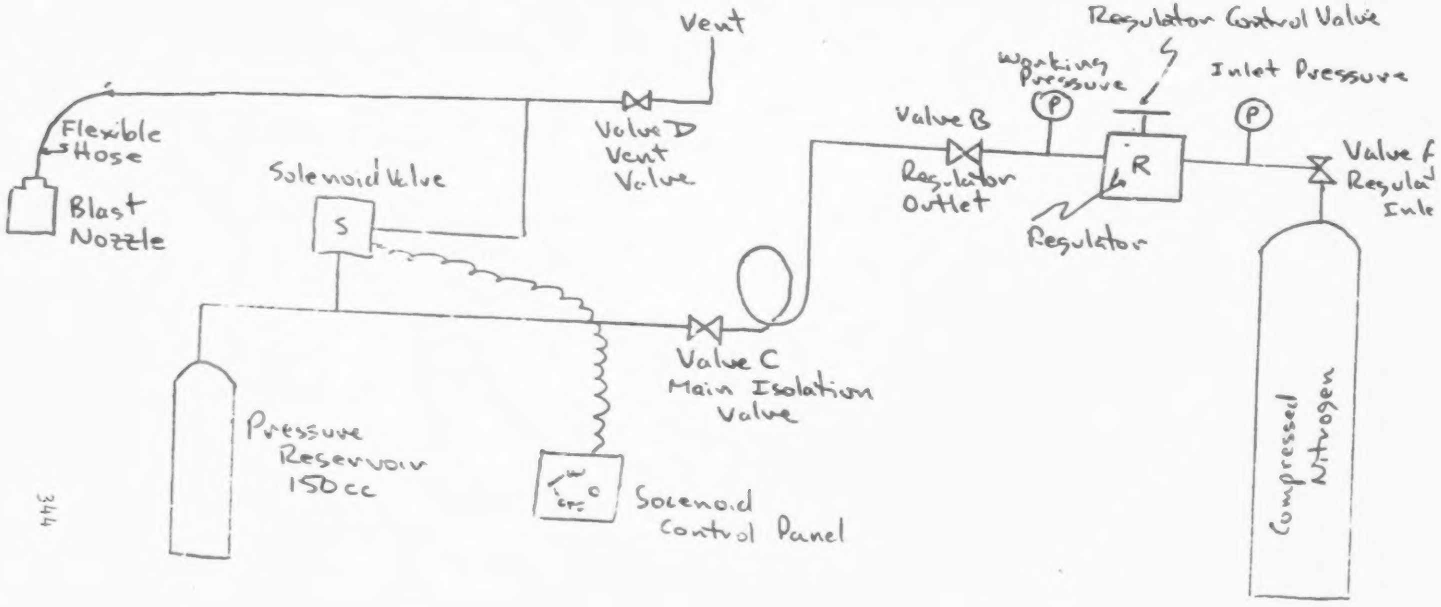
Exploratory Laparotomy - brief description -

Post Mortem - Brief description:

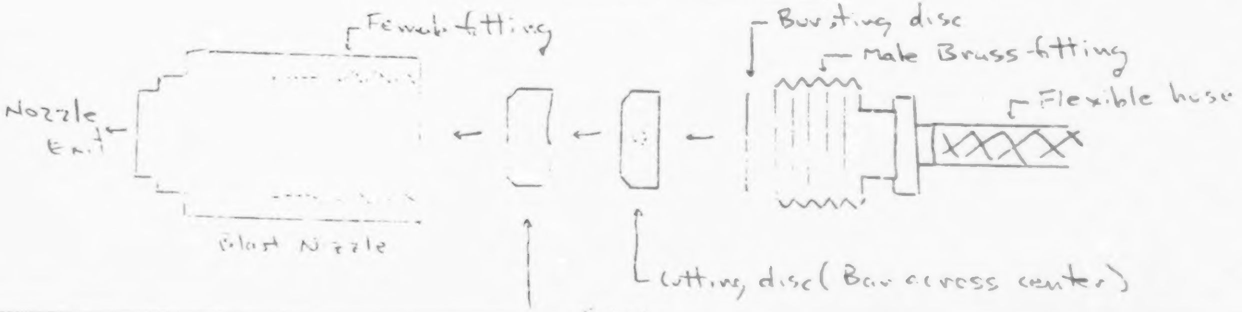
References:

1. Clemedson, C-J. An Experimental Study of Air Blast Injuries. Acta Physiol. Scand., 18: (Suppl LXI) 1-200, 1949.
2. Bowen IG, Fletcher ER, Richmond DR. Estimates of Man's Tolerance to the Direct Effects of Air Blast. Defense Atomic Support Agency Report DASA-2113, 1968.
3. Chiffelle, TL, Pathology of Direct Air-Blast Injury. DASA-1778, 1966.
4. White LS, Jones RK, Damon EG, Fletcher ER, Richmond DR. The Biodynamics of Airblast, Defense Nuclear Agency Report DNA 2738T, 1 July 1971.
5. Harmon JW, Haluszka M. Care of Blast-Injured Casualties with Gastrointestinal Injuries. Mil. Med. 148(7), 586-588, July 1983.
6. Final Pahtology Report. Blast Overpressure Project (WRAIR Protocol 005-80). July 1980.

BLAST GENERATOR - Schematic "A"


Vent
Regulator Control Valve
Working Pressure
Inlet Pressure
Flexible Hose
Value D Vent Valve
Value B Regulator Outlet
R
Value A Regula Inle
Blast Nozzle
Solenoid Valve
S
Regulator
Value C Main Isolation Valve
Pressure Reservoir 150cc
Solenoid Control Panel
Compressed Nitrogen

BLAST NOZZLE ASSEMBLY - Schematic "B"


Female fitting
Bursting disc
Male Brass fitting
Flexible hose
Nozzle Exit
Blast Nozzle
Cutting disc (Bar across center)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA OG 6761	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	CD	228 WWI7
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/2			

11. TITLE *(Precede with Security Classification Code)* (U) Regulatory Mechanisms and Pathophysiology of Hematopoiesis Application to Military Hematology

12. SUBJECT AREAS

0611 Life Support 0603 Biology 0605 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In-house

17. CONTRACT GRANT

a. DATE EFFECTIVE

EXPIRATION

b. CONTRACT/GRANT NUMBER

c. TYPE

d. AMOUNT

e. KIND OF AWARD

f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	5.0	184
85	5.2	290

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Medicine
b. ADDRESS *(include zip code)* Washington, DC 20307-5100	b. ADDRESS Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR WRIGHT, D G
d. TELEPHONE NUMBER *(include area code)* (202)-576-3551	d. TELEPHONE NUMBER *(include area code)* (202)-576-3358
21. GENERAL USE FINA MILITARY CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* SALVADO, A J g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* MEAGHER R

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Leukocytes; (U) Bone Marrow; (U) Hematopoiesis; (U) Marrow Failure; (U) Erythrocytes (U) RAM III; (U) Rabbits; (U) Mice

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) To define the hematologic pathophysiology of bone marrow toxicity from certain families of chemical agents, drugs, radiation, and acute infection; to identify modalities that may protect against hematopoietic stem cell injury; to study basic mechanisms involved in the regulation of hematopoiesis, including iron absorption and to define and purify hematopoietic regulatory mediators. A basic understanding of the regulation of hematopoiesis is very important to the military because of numerous marrow toxic conditions (radiation, drugs, infections, chemicals) to which military personnel may be exposed during their duties.

24. (U) Experimental procedures include biochemical and cell culture techniques, animal models, and the isolation of normal human bone marrow cells. Studies also involve electron microscopic analysis of the ultrastructure of bone marrow tissue during its morphogenesis.

25. (U) 83 10 - 84 09 Studies of human in vitro long-term marrow cultures using a microtized system have examined the effects of temperature, feeding schedule, serum source, oxygen tension and return of a portion of the cells during weekly depletion and feeding. Using this system other studies have focused on definition of stromal cells that form the "hematopoietic micro-environment" in vitro. Stromal cells have been grown in a serumless culture system to facilitate examination of production of critical regulatory substances by the stromal cells. The growth of such stromal cells in cultures which have been irradiated with or without the addition of autologous density separated hematopoietic progenitors has been studied by scanning electron microscopy and time lapse microcinematography. Methods for purification of erythropoietin (one physiologic regulator) from human urine or a murine cell line have been scaled up to a preparative level. For technical report, see Walter Reed Army Institute Research Annual Report 1 Oct 83 - 30 Sep 84.

DD FORM 83 MAR 1498

EDITION OF MAR 68 IS OBSOLETE.

Project 3M161102BS10: RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 228 Hematopoiesis Regulatory Mechanisms and Pathophysiology of Application to Military Hematology

Investigators LTC Daniel Wright, MC; LTC August Salvado, MC; COL William Crosby, MC; Ms. Mary Cutting, MS (Fellow, GWU); Dr. Richard Meagher (GS 12)

Description

Blood cells constitute a complex organ of which normal function requires continuous self-renewal of blood precursor cell within the bone marrow. The demands of blood cell renewal (hematopoiesis) are enormous, and for this reason hematopoiesis is particularly sensitive to the toxic effects of chemicals, drugs, radiation, and acute infections which interfere with cell division or differentiation. The objectives of this work unit are to study basic mechanisms involved in the regulation of hematopoiesis using tissue culture of stem cells and committed hematopoietic precursor cells from human, mouse, and rabbit marrow, using leukemic cell lines, and using allogenic transplantation of bone marrow tissue in animals.

Specific studies are designed to study the effects of mediators derived from mature leukocytes and inflammatory fluids upon hematopoiesis, to study the biochemistry and physiologic effects of erythropoietin upon stem cell maturation, to study basic mechanisms by which iron absorption is regulated and by which iron is utilized by hematopoietic tissues, and to develop *in vitro* tissue culture techniques which will permit the maintenance and expansion of human pluripotent stem cells capable of effecting hematopoietic reconstitution in a recipient with bone marrow failure.

Progress

1. Studies of long-term in vitro culture of normal human bone marrow

A. Development and optimization of a miniaturized system for human long-term bone marrow culture.

The development of a culture system which offers the capability of direct examination of the adherent stromal cells or microenvironment is critical for the study of hematopoiesis in vitro. Employing a Leighton culture tube which has a removable 5 cm^2 coverslip, we have developed a miniaturized Dexter culture system that offers several advantages to previously described marrow culture techniques. The first advantage is the capability of direct examination of the stromal cells without disturbing the cellular architecture, thereby affording us the opportunity to study the cell types that comprise the adherent layer and to study possible cell-cell interactions in a more rigorous fashion. Further, we have devised a "recharge" system to aid us in optimizing the Leighton tube method that allows us to quantitate the net production of cells from these cultures. Following an initial four weeks of culture that is necessary for the development of an adherent stroma the cultures are irradiated with 800 Rads of gamma irradiation and then "recharged" with 10^7 autologous human marrow cells. The fresh innoculum of marrow cells provides a source of pluripotent stem cells which occupy the "preformed niches" in the adherent stroma. Following the "recharge" it is possible to monitor the production of cells from the cultures for 4-5 weeks post recharge. Also, it has been possible to perform electron microscopic studies on the adherent stromal cells since they are directly accessible, as compared to conventional flask cultures.

Since we have microtized the system by using Leighton tubes we have reduced the quantity of nutrient media required thereby lowering the cost of performing long-term marrow culture. Another advantage that the Leighton tube method offers is the possibility of studying normal hematopoiesis in vitro using photocinematography since the Leighton tubes can be placed in mini-incubators that we have devised and observed for 2-3 day periods under normal conditions.

B. Definition of stromal cells and nutritional requirements

The adherent layer of "stromal" cells which forms on the bottom of the culture flasks during the first 2-4 weeks of bone marrow culture is critical for supporting the survival and proliferation of hematopoietic stem cells. To better evaluate the production of regulatory substances and define the cells which produce them we have begun to establish a serum free culture system in which the critical nutritional requirements are completely defined. Aside from the basic medium, salts and vitamins, it has been determined that a source of lipid in the form of lipoprotein fractionated from horse serum by ultracentrifugation is needed. Along with this, the addition of the proteoglycan heparin was required for the stromal cells to become adherent. The above conditions allowed for the establishment of a monolayer of adherent "stromal" cells which are morphologically different from serum fed controls and are likely a subset of "stromal" elements. The same conditions also allow for the survival of committed hematopoietic cells for at least 2 weeks. The stromal cells which develop under serum free conditions are being characterized by histochemical stains as well as biological function (production of tissue plasminogen activator, factor VIII antigen, angiotensin converting enzyme, and prostaglandins).

2. Studies of erythropoietin (Ep)

Studies continue on the use of high performance liquid chromatography (HPLC) for the purification of the erythroid regulatory hormone Ep. Analytical column techniques have established the feasibility for rapid, high yield, high specific activity purification of this substance from human urine. Recombinant DNA techniques and the establishment of cell lines which constitutively produce Ep, however, will soon lead to cleaner and more readily available sources of the hormone in large volumes of culture media but which will also be contaminated with media components and bacterial or cellular metabolic products. We have begun to extrapolate our analytical procedures to the development of preparative HPLC methods for rapid, high efficiency purification of Ep. This includes: a) reverse phase column chromatography using ethonol gradients which have been well established in our laboratory on analytical columns and b) the addition of ion exchange chromatography on a preparative DEAE column. Although Ep does not separate on an HPLC hydroxyapetite column as expected from the literature data using macro columns, work continues to find suitable conditions for this separation.

3. The effects of ethanol upon bone marrow progenitor cells.

Ethanol is a toxic substance that directly or indirectly affects cellular function in every organ of the body. Within the bone marrow no cell line is impervious to the deleterious effects of ethanol. Acute ethanol ingestion causes vacoules in erythroid and myeloid progenitor cells and causes thrombocytopenia in the megakarocyte compartment. Folate metabolism is impaired and chronic ethanol ingestion interferes with iron utilization and can lead to megaloblastic hematopoiesis. The mechanism of ethanol-induced toxicity upon the bone marrow is not well understood. We are investigating the in vivo and in vitro effects of acute and chronic ethanol ingestion upon bone marrow using a well-defined animal model of alcoholism. We are employing clonal assays for the committed hematopoietic progenitors of the erythroid and myeloid series, namely, the early and late erythroid progenitors (CFU-E and BFU-E) and the myeloid progenitor (CFU-GM) to investigate the effects of ethanol upon the hematopoietic system. Further, we are studying the effects of pyridoxine supplementation of alcoholic diets to determine if there are any beneficial effects of pyridoxine supplementation upon hematopoiesis.

4. Studies of the regulation of iron absorption

We have completed studies of iron absorption kinetics using a low-dose oral iron tolerance test in which serial plasma iron levels are used to follow iron absorption. This technique, described in the FY 83 annual report, has been used to define the effects of oral antiacids on the absorption of dietary levels of iron. The technique has also been used to define the duration of the refractory period for further iron absorption that follows an initial dose of 10-20 mg. Finally the changes in plasma iron changes after low dose oral iron have been quantitatively related to iron absorption as measured by radio-iron tracer studies.

Future plans

Studies of the regulation of hematopoiesis using in vitro systems for the long-term culture of human bone marrow, and of hematopoietic growth factors will continue in FY 85. Attention will be focused on the definition of critical stromal elements established in a serum free system which can support hematopoietic proliferation and/or differentiation. Mechanisms for establishment of a hematopoietic microenvironment as well as support and regulation of hematopoiesis will be sought. Development of completely defined tissue culture systems for the maintenance and expansion of human pluripotent, hematpoietic stem cells will remain a central developmental goal.

Abstracts

1. Salvado AJ, Meagher RC, Young P. Meierovics AI, Wright, DG: Effects of serum free culture conditions upon hematopoietic and stromal cells in human long-term marrow cultures. J Cell Biochem (Supplement:8A:709, January 1984). (Presented at UCLA conference on Molecular Biology, March 1984).

2. Salvado AJ, Meagher RC, Young P, Solem MA, Meierovics AI, Wright DG: Serumless media with added lipoprotein supports

hematopoietic stem cell survival in human long-term marrow culture but not the establishment of an adherent stroma. Clin. Res. 32:322A, April 1984.

3. Salvado AJ, Meagher RC, Solem MA, Meierovics AI, Wright DG: Establishment of an adherent stroma in serumless human long-term marrow cultures. Expematol 12(6):456, 1984. (Presented at The International Society for Experimental Hematology Meetings, August 1984).

4. Meagher RC, Salvado AJ, Solem MA, Meierovics AI, Wright DG: A miniaturized Dexter culture system for the study of human hematopoiesis in vitro. Exp. Hematol. (Presented at The International Society for Experimental Hematology Meeting, August 1984). Exp. Hematol. 12(6):361,1984.

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6. Wright DG, Meagher RC, Salvado AJ: Micro-cinematographic studies of human bone marrow in long-term dexter cultures. Blood (in press), 1984.

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1. Meagher RC, Khouri JA, Derelanko MJ, Kruger RE, Rothman SA, LoBue J, and Gordon AS. Mechanism underlying the induction of erythrocytosis by nickel subsulfide (in review), 1984.

2. Wright DG, and Greenberger JS, eds.: Long-term Bone Marrow Culture, AR Liss, NY, NY, 1984 (in press).

3. Salvado, AG, Meagher RC, and Wright DG: The differentiation of hematopoietic cells in long-term bone marrow culture, in: Long-term Marrow Culture, Wright DG, and Greenberger JD, eds., A.R. Liss, NY, NY, 1984 (in press).

4. Meagher RC, Sieber F, Spivak JL: Susceptibility to merocyanine 540 - mediated photosensitization: A differentiation marker on murine hematopoietic progenitor cells. J. Cell Physiol. 116:118-124, 1983.

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6. Crosby WH: Structure and function of the spleen. Chapt 14 in Williams WJ et al. (eds); Hematology, ed 3. New York, McGraw Hill, 1983, pp 89-97.

7. Crosby WH: Hypersplenism. Chapt 75, in Williams WJ et al (eds): Hematology, ed 3. New York, McGraw Hill, 1983, pp 660-666.

8. Crosby WH: The golden age of the Army Medical Corps: A perspective from 1991. Military Med. 148:707-711, 1983.
9. Crosby WH, Beutler E, Freedman ML: Pale blood, thin blood. Emergency Med. 15, 11:26-47, Dec, 1983.

10. Crosby WH: Landmark perspective. Pernicious anemia. A commentary on Minot GR, Murphy WP: Treatment of pernicious anemia by a special diet. JAMA 1926; 87:470-476. JAMA 250:3336-3338, 1983.

11. Markovitz A, Crosby WH: Chemical carcinogenesis. A soil fumigant, 1, 3-dichloropropene, as a possible cause of hematologic mahgnancies. Arch. Intern. Med. 144:1409-1411, 1984.

12. Crosby WH: Chapter 5 Hematologic Diseases in Hunter's Tropical Medicine, 6th Ed., ed by Strickland GT. Philadelphia, WB Saunders Co, 1984, PP 33-40.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DAOG6762
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AJ	229 WWI3
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 82/83-6.2/1			

11. TITLE (Precede with Security Classification Code)
(U) Military Hematology

12. SUBJECT AREAS
0611 Life Support 0603 Biology 0605 Clinical Medicine

13. START DATE: 58 05
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-house

17. CONTRACT GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	5.0	539
85	7.8	423

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
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20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
Division of Medicine
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c. NAME OF PRINCIPAL INVESTIGATOR
WRIGHT, D G
d. TELEPHONE NUMBER (include area code)
(202)-576-3358
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
ALVING, B A
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE
FINA
MILITARY CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Mice; (U) Rabbits; (U) Monkeys; (U) Coagulation; (U) Hematopoiesis; (U) Blood; (U) Marrow Failure; (U) Erythrocytes; (U) Leukocytes; (U) Volunteers.(U) Lab Animals;(U)RAM

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To define the hematologic Pathophysiology of trauma, infections, shock, marrow toxic drugs or radiation as related to diseases of military importance; to identify modalities to restore hemostasis, to augment host defense systems against infection. The importance of this basic research to the military is wide ranging and is applicable to both health maintenance of military personnel exposed to unusual environmental, toxic and infectious hazards but also to the treatment of militarily relevant disease.

24. (U) Experimental procedures include biochemical, immunologic, and cell culture methods; in vitro cell-free and membrane-dependent systems; large and small laboratory animal models; and studies of human subjects.

25. (U) 83 10 - 84 09 a) Experimental clinical studies have been done on the regulation of vitamin K dependent clotting systems in normal subjects, patients receiving warfarin anticoagulation, and a kindred with heritable warfarin resistance. b) Animal studies have explored the relationship between circulating fibrinogen levels and the systemic release of fibrinolytic mediators, plasminogen activator and tissue factor. c)Clinical studies of clotting factor replacement and of acquired dysfibrinogenemia syndrome were done. d) Continued studies of myeloid leukemia cell lines that may be induced to differentiate in vitro and of in-bred dogs with congential cyclic neutropenia and marrow regulatory dysfunction have confirmed previous findings of work-unit concerning relationships between purine nucleotide metabolism and the regulation of terminal myeloid cell differentiation. e) A human neutrophil secretory glycoprotein that stimulates monocytes/macrophage functions has been further characterized biochemically and partially purified. f) The relationship between essential fatty acid deficiency and abnormal phagocyte function has been further explored in a cell culture system. For technical report, see Walter Reed Army Institute of Research Annual Report, 1 Oct 83 - 30 Sep 84.

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Project 3M161102BS10: RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 229 Military Hematology

Investigators LTC Daniel G. Wright, MC; LTC Barbara Alving, MC; COL John Kark, MC; Mr. Charles Barr, GS-12; COL William Crosby, MC; LTC August Salvado, MC; Dr. Jan Palmblad (NRC Senior Fellow); MAJ Robert Knight, MC (WRAMC); Dr. Richard Meagher, GS-12; (WRAMC); Dr. Chitra Krishnamurta, GS-12; Dr. R. Lange (visiting professor, Univ. Tennessee).

Description

Two distinct research areas have been explored under this work unit in FY84.

1. Studies of coagulation and plasma proteins

The balance between blood coagulation factors, enzymes of the fibrinolytic system and their inhibitors is essential in determining if a person who has undergone acute injury (trauma) will have a severe problem with bleeding. Studies in this lab are currently directed toward defining the levels of coagulation factors that appear to be necessary for hemostasis in patients with single and multiple factor deficiencies.

In addition we are using a rabbit model to define the agents and clinical conditions (shock, trauma) that stimulate release from the endothelium of plasminogen activators which are responsible for causing clot lysis through activation of plasminogen to plasmin. These agents may also stimulate simultaneously release of inhibitors of the fibrinolytic system. Therefore the degree of excessive clot lysis that occurs after release of plasminogen activators depends on the balance between the activators and their inhibitors.

2. Studies of blood phagocytes

Phagocytic blood leukocytes are critical to host defenses against bacterial and fungal infections and to the development and outcome of inflammatory responses. Studies of human neutrophil and monocyte function have concentrated upon understanding the secretion of soluble mediators by these cells that influence the immunoresponsive functions of macrophages and lymphocytes and affect connective tissue disorganization and repair. Studies have also been directed at understanding factors that regulate the production of neutrophils in the bone marrow and that influence the distribution, utilization, and function of these phagocytes in peripheral tissues.

Progress

1. Studies of coagulation and plasma proteins

A. Clinical coagulation studies

We have initiated a clinical study that involves monitoring levels of coagulation factors as well as inhibitors (e.g. anti-thrombin III) in patients before and after coronary bypass surgery. We are correlating these data with their platelet counts and bleeding diathesis. The purpose of the study is to determine levels of factors that appear to provide hemostasis in the surgical situation and to use blood products on a more rational basis. We have also completed a clinical study of a patient congenitally deficient in factor X only. By following this patient through multiple bleeding episodes (and treating with plasma) we have been able to determine the levels of factor X needed for hemostasis.

B. Studies of Plasminogen Activators in a rabbit model

During the past year we have studied agents that induce release of PA (plasminogen activator) in the rabbit. Before initiating these studies we developed a sensitive chromogenic assay for measurement of small amounts of PA in plasma samples. We also developed a method of fibrin plate zymography by which we can assess the molecular weight of plasminogen activators by first subjecting them to SDS-gel electrophoresis and them by overlaying the gels on fibrin plates that are rich in plasminogen and observing areas of lysis.

Two different studies in rabbits are currently in progress. In one study we are attempting to determine the effect of *in vivo* non-cross linked fibrin formation on release of PA. We have infused rabbits with ancrod (venom from the Malayan pit viper) which causes conversion of fibrinogen to fibrin. The animals become fibrinogenemic and have high titers of fibrinogen-fibrin degradation products. They have no thrombotic complications, presumably because PA is released. We are studying the level and type of PA released and will determine if the conversion of fibrinogen to fibrin is the trigger for its release.

In a second study we are measuring PA release after infusion of DDAVP (an analogue of vasopressin). The drug is now used clinically to stimulate release of von Willebrand factor from endothelial cells. We will determine if PA release after DDAVP is sufficient to cause an increased rate of lysis of clots that will be formed *in vivo*.

2. Studies of blood phagocytes

A. The regulation of myeloid cell maturation

In our previous studies with the human promyelocytic leukemic cell line, HL-60, we defined changes in purine metabolism that consistently occur with induced differentiation of these immature myeloid cells. When HL-60 cells are exposed to compounds that induce maturation, biosynthesis of guanylates from the central intermediate, IMP, is reduced and intracellular guanosine nucleotide (NTD) pools shrink. Furthermore, our studies suggested that these metabolic changes involve the down-regulation of the enzyme IMP dehydrogenase (IMPD), leading us to discover that specific inhibitors of IMPD, such as mycophenolic acid and 2--ribofuranosylthiazole-4-carboxamide (RTC), are potent inducers of HL-60 cell maturation. We then demonstrated down regulation of IMPD by direct measurements of this enzyme, lending further support to our evolving concept that IMPD and the regulation of guanosine NTD supplies may be critical to the regulation of myeloid cell differentiation.

We have now confirmed these observations in an unrelated myeloid cell line, RDFD-2, which we isolated from a patient at WRAMC with acute myelogenous leukemia, and in studies with an *in vivo* model, canine cyclic neutropenia. In this hereditary disease of dogs, myelopoiesis proceeds in waves, not in a normal continuous fashion. Repeated marrow aspirates were obtained from cyclic neutropenic dogs and from normal littermates. Nucleated marrow cells, cleared of red cells and enriched for myeloid elements, were prepared with density gradients. Measurements for NTD concentrations and IMPD levels in extracts of these cells demonstrated fluctuations of guanosine NTD and IMPD levels in marrow cells of CN dogs, unlike the stable measurements observed in normal animals. As we would have predicted from our previous *in vitro* studies, guanosine NTD and IMPD levels were observed to fall as the wave of myelopoiesis proceeded through the differentiation phase of the myelopoietic cycles.

B. Studies of neutrophil function in essential fatty acid deficiency

A Model of essential fatty acid deficiency (EFAD) was developed in monkeys in order to study its effects upon the host defense functions of neutrophils. The essential fatty acid, linoleic acid, is the obligatory precursor of arachidonate. Archidonic acid is incorporated into the physpholipid constituents of cell membranes and appears to be very important in propogating receptor mediated signals that stimulate cells to carry out their specific normal functions. Arachidonate metabolism appears to be particularly important in the stimulus-response coupling of neutrophils, which are the principal blood phagocytes. Linoleic acid deficiency was produced in monkeys by total intravenous alimentation using solutions that delivered carbohydrate calories, and vitamins but no fat. Monkeys receiving fat-free IV nutrition were compared with control animals that received the same preparations but with fat emulsions (intra-lipid) added. These experimental conditions are analogous to those of post-surgical or post-trauma patients who must receive all their nutrition intravenously. In monkeys not given lipid, biochemical evidence for essential fatty acid deficiency was clearly evident by one week and progresed during the subsequent 2 weeks of three week experimental periods. While marked decreases in plasma lipid and leukocyte membrane lipid linoleic acid levels occurred first, by 2 1/2 weeks there was also a significant decrease in leukocyte membrane arachidonate levels.

Several different monkey species were evaluated in these studies before finding that the African Green monkey was the best for study by being most able to tolerate the experimental model, and by having neutrophils with separation characteristics most similar to humans. We then demonstrated with this model that inflammatory functions of arachidonate-deficient monkey PMNs (e.g. chemotaxis and stimulated oxidative metabolism) is deficient - the functional abnormalities appearing coincident with the fatty acid deficiency, and disappearing with its correction by the replacement of lipid in the TPN diets.

A second *in vitro* model of EFAD confirmed the relationship between cellular arachidonate levels and phagocytic cell functions. We observed that we could render the human myeloid cell line, HL-60, completely deficient in linoleic acid and arachidonic acid by culturing these cells in defined, serum-free media. These cells can be induced to express certain

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functions of mature phagocytes, such as chemotaxis. We observed that the specific replacement of linoleic acid, which is readily metabolized to form arachidonate by the cells, caused a marked enhancement of the functional responsiveness of the cells. These studies further strengthened our basic observation and concept that changes in cellular arachidonate levels, as may occur in TPN and other forms of nutritional deprivation, can influence phagocytic cell function and the inflammatory response.

Future Plans

Studies of coagulation and plasma proteins and of blood phagocytes will continue in FY85 along lines of work carried out in the past three years. These studies will continue to include investigations in to the character and function of glycoproteins of human neutrophil secondary granules (discussed in previous annual reports under this work unit) on inflammatory cell function and on the regulation of granulopoiesis.

Abstracts

1. Liu, Y.P., Alving, B.M., Lucas, D.L., Wright, D.G. Stimulated production of plasminogen activator during induced maturation of the human promyelocytic leukemia cell line HL-60. Blood 62, 151a, 1983.

2. Baldwin, P., Richard R., Alving, B. A dilute phospholipid APTT assay: increased sensitivity for verification of lupus anticoagulants. Blood 62, 280a, 1983.

3. Wright, D.G., Palmblad, J.E., Salem, N.: Essential fatty acid deficiency (EFAD) and phagocyte function: Studies with the HL-60 cell line. Blood (in press) 1984.

4. Palmblad, J.E., Wannemacher, R.M., Salem, N., Wright, D.G.: Essential fatty acid deficiency (EFAD) and neutrophil function. Clin. Res. 32:378A, 1984.

5. Wright, D.G., Jones, J.B., Lucas, D.L., Knight, R.L., Lange, R.D.: Cycling purine nucleotide pools in bone marrow cells of dogs with cyclic neutropenia. Clin. Res. 32:326A, 1984.

6. Wright, D.G., Palmblad, J.E., Salem, N.: Essential fatty acid deficiency and phagocyte function: Studies with the HL-60 cell line. Blood (in press), 1984.

AVAILABLE

Published Articles

1. Marks, E., Alving, B. anmd Pisano, J.J.: The kallikrein-kinin system in the brown Norway rat. Thromb. Res. 31, 653-656, (1983).

2. Alving, B.M., Barr, C.F., and Tang, D.B. L-asparaginase: acute effects on protein synthesis in rabbits with normal and increased fibrinogen production. Blood 63, 823-827, (1984).

3. Alving, B.M., Niebyl, J.R., Proud, D., Mason, B.L., Pisano, J.J. Human plasma prekallibrein and high molecular weight kininogen decrease during parturation. Thromb. Res. 34, 473-77, (1984).

4. Olson, T.A., Alving, B.M., Chesier, J.L., Landes, R.D., and Ruymann, F.B. Intracerebral and subdural hemorrhage in a neonate with Hemophilia A. Am J Pediatr Hematol/Oncology (in press).

5. Alving, B.M., Strickler, M.P., Knight, R.D., Barr, C.F., Berenberg, J.L., Peck, C.C. Hereditary warfarin resistance: investigation of a rare phenomenon. Archives Int. Med. (in press).

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7. Dawson, N.A., Barr, C.F., Alving, B.M. Acquired dysfibrinogenemia: a paraneoplastic syndrome in renal cell carcinoma. Am J Med (in press).

8. Lucas, D.L., Webster, H.K. and Wright, D.G.: Purine metabolism in myeloid precursor cells during maturation: Studies with the HL-60 cell line. J. Clin. Invest. 72:1889-1900, 1983.

9. Lucas, D.L., Robins, R.K., Knight, R.D. and Wright, D.G.: Induced maturation of the human promyelocytic leukemia cell line, HL-60, by 2- -D-ribofuranosy selenazole-4-carboxamide. Biochem. Biophys. Res. Comm. 115:971-980, 1983.

10. Chaing, P.K., Lucas, and Wright, D.G.: Induction of differentiation of HL-60 human promyelocytic leukemia cells by 3-deaza purines. Ann. N.Y. Acad. Sci. (in press), 1984.

11. Lucas, D.L., Tanuma, S., Davies, P.J.A., Wright, D.G., and Johnson, G.S.: Maturation of human procyelocytic leukemia cells induced by nicotinamide; Evidence of a regulatory role for ADP-ribosylation of chromosomal proteins. J. Cell Physiol. (in press), 1984.

12. Wright, D.G.: Leukocyte Transfusion: Thinking Twice. Am. J. Med. 76:637-644, 1984.

13. Wright, D.G.: Leukocyte Transfusions: in: Infections Complications of Neoplastic Disease: Controversies in Management, A.E. Brown and D. Armstrong, eds., Yorke, N.Y., N.Y., 1984 (in press).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OG 9284
2. DATE OF SUMMARY: 84 10 01

REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	6 1102A	3M161102BS10	AH	230 WWH5
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code): (U) Biological Roles of Surface Membrane Components: Parasite Model Systems

12. SUBJECT AREAS: 0601 Biochemistry 0603 Biology

13. START DATE: 81 10
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT/GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.5	459
85	4.0	507

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code): Washington, D.C. 20307 - 5100
c. NAME OF RESPONSIBLE INDIVIDUAL: TOP, F H Jr
d. TELEPHONE NUMBER (include area code): (202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS:
Division of Biochemistry
Washington, D.C. 20307 -5100
c. NAME OF PRINCIPAL INVESTIGATOR: Olenick, J G
d. TELEPHONE NUMBER (include area code): (202)-576-3017
f. NAME OF ASSOCIATE INVESTIGATOR (if available): Hansen, B D
g. NAME OF ASSOCIATE INVESTIGATOR (if available): Geller, R

21. GENERAL USE: FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Surface Antigens; (U) Gene Cloning; (U) Transport Receptors; (U) 2-D Gel Analysis; (U) RAMI

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objective is to investigate the membrane surface components of parasites with a view to elucidate surface associated biochemical processes. This will afford the development of immunoprophylactic and/or chemotherapeutic protection of military personnel against tropical diseases of military importance.

24. (U) Surface antigens are studied by application of recombinant DNA, gene cloning, restriction analysis and probe hybridization techniques. Radiolabeled ligands are employed to determine transport processes and surface receptors. 2-D gel electrophoresis is used to characterize surface antigens of isolated membranes.

25. (U) 83 10 - 84 09. Specific binding of a radiolabeled adenosine receptor ligand cyclohexyladenosine to purified promastigote membranes of Leishmania mexicana mexicana was determined. Membranes exposed to increasing concentrations of the ligand showed a significant decrease in adenylate cyclase activity while in whole cells cAMP levels were reduced and the rates of proliferation and transformation to the amastigote form increased. Whole cell protein components from different developmental stages of Leishmania panamensis were analyzed by 2-D gel electrophoresis. Protein patterns of each stage were distinctive but contained several protein spots that appeared to be invariant. Translation products from Leishmania donovani mRNA were analyzed using human antisera as well as antisera from challenged and rechallenged animals. Monoclonal antibodies were raised against purified membrane components of L. donovani. These monoclonals are being used in conjunction with the aforementioned antisera to probe cDNA and genomic banks constructed in two dissimilar expression vector systems. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

PROJECT: 3M161102BS10 RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

WORK UNIT: 230 Biological Roles of Surface Membrane Components: Parasite Model Systems

INVESTIGATORS:

Principal: John G. Olenick, Ph.D.
Associate: Ruth Geller, Ph.D.; Brian D. Hansen, Ph.D.; CPT Ruthann M. Smejkal, Ph.D.
Assistant: SP4 Lynn Decker; SP5 Jose Perez-Arbelo; SP4 William M. Schweitzer

The objective of this work unit is to investigate the membrane surface components of parasitic protozoa with a view to elucidate surface-associated biochemical processes. This will afford the development of immunoprophylactic and/or chemotherapeutic protection of military personnel against leishmaniasis and/or other tropical diseases of military importance. The following investigations were conducted:

1. Adenosine receptor binding in purified promastigote membranes of *Leishmania spp.*

2. Nucleoside transport in promastigotes and amastigotes of *Leishmania mexicana mexicana*.

3. Fluorogenic substrate detection of viable intracellular and extracellular pathogenic protozoa. (In collaboration with the Dept. Immunology, DCD&I).

4. Two-dimensional gel electrophoresis analysis of whole cell protein components from different developmental stages of *Leishmania braziliensis panamensis*.

5. Analysis of translation products from *Leishmania donovani* mRNA with various antisera. (In collaboration with the Dept. Immunology, DCD&I.)

6. Use of antisera and monoclonal antibodies to probe cDNA and genomic banks constructed in two dissimilar expression vector systems.

1. Adenosine receptor binding in purified promastigote membranes of *Leishmania spp.*

Leishmania spp. are unable to synthesize purines *de novo* and must acquire these essential nutrients from the host organism. The purine nucleoside adenosine is readily taken up and incorporated into nucleic acid by both the promastigote and amastigote stages of the parasite. However, our recent studies suggest that adenosine may also effect intermediary metabolism of the parasite when bound to an adenosine receptor on the external surface of the plasma membrane. Initial experiments measured the total, nonspecific and specific binding of a nonmetabolizable adenosine receptor ligand (^{3}H-cyclohexyladenosine) on purified promastigote membranes over increasing incubation times from 15 seconds to 30 minutes. Since specific binding equilibrated within 20 minutes, all subsequent incubations were conducted for 30 minutes. Specific and nonspecific binding at various concentrations (0-800 nM) of the radio-labeled adenosine receptor ligand were determined. Scatchard plots of the specific binding data yielded a Kd value of approximately 0.1 nM. Adenosine receptor binding in the mammalian neuron is closely linked to the regulation of membrane adenylate cyclase activity. This enzyme activity was also demonstrated in our promastigote membrane preparation. Moreover, adenylate cyclase activity was significantly inhibited in the presence of the nonlabeled adenosine receptor ligand, cyclohexyl-adenosine. Therefore, preliminary data indicate that adenosine binding to the membrane receptor inhibits cyclic AMP synthesis and may ultimately control cell replication and transformation.

2. Nucleoside transport in promastigotes and amastigotes of *Leishmania mexicana mexicana* (WR 227).

Due to the absence of *de novo* purine synthesis in *Leishmania spp.* and the necessary acquisition of the purine ring from the host organism, membrane transport systems must be operating. The purpose of the present study was to characterize kinetically the membrane transport of two nutritionally important nucleosides, adenosine and guanosine, in both the mammalian (amastigote) and insect (promastigote) forms. A rapid sampling assay was developed using nitrobenzylthioinosine to terminate short interval incubations measuring nucleoside transport and centrifuging cells from radiolabeled media through silicone oil. When uptake of ^{14}C-adenosine and ^{14}C-guanosine was measured as a function of time, a linear relationship for the first two minutes was demonstrated, suggesting first order rate kinetics during initial transport. However, to minimize the effects of metabolism all incubation periods were limited to five seconds. High performance liquid chromatography analysis revealed that, within this time interval, less than 3% of radiolabeled adenosine or guanosine was metabolized. Velocity of adenosine and guanosine transport measured as a function of substrate concentration was also reported with Vmax values of 29 nM and 55 nM and Kt values of 3.5 μM and 1.5 μM, respectively. Competitive interactions measured among nucleosides utilized by the parasite were conducted. These data indicate a maximum of two purine nucleoside transport loci on the promastigote membrane; one specific for adenosine and a second transporting all other nucleosides. This specific transport locus for adenosine may be closely associated with 5' nucleotidase activity found on the external promastigote membrane.

3. Fluorogenic substrate detection of viable intracellular and extracellular pathogenic protozoa. (In collaboration with the Dept. Immunology, DCD&I.)

Cell viability assays, important in research with pathogenic protozoa, have included *in vitro* cultivation, animal infectivity, vital dye staining and radiolabeled substrate uptake. These procedures can be slow, difficult to interpret, dangerous or expensive. In the present study, the non-fluorescent compounds, fluorescein diacetate (FDA), 5-carboxyfluorescein diacetate (5-CFDA), 6-carboxyfluorescein diacetate (6-CFDA) and ethidium bromide (EB), were used in a rapid fluorescence microscope procedure to detect viable parasitic protozoa. Living cells, relatively impermeable to EB, rapidly metabolized FDA, 5-CFDA and 6-CFDA to fluorescent fluorescein or its carboxy derivatives. These products caused living cells to fluoresce yellow-green under ultraviolet light (UV). Dead or dying cells stained rapidly with EB and fluoresced red under UV light when EB complexed with nucleic acids. The EB/FDA procedure detects viable Acanthamoeba, Entamoeba, Giardia, Trypanosoma, Babesia, Leishmania and Plasmodium. Intra- and extra-cellular viable P. falciparum stained with FDA and 5-FDA, while 6-FDA only detected viable extracellular parasites. This method affords a faster, more reliable indication of amastigote viability within the macrophage than is possible using in vitro cultivation or giemsa staining.

4. Two-Dimensional gel elecrophoresis analysis of whole cell protein components from different developmental stages of Leishmania braziliensis panamensis.

Leishmania, like other protozoan parasites, exists in different developmental stages in its mammalian host and insect vector. The promastigote (the insect vector form) grows well in culture, while the intracellular amastigote (the mammalian host form) is difficult to obtain in large numbers. The promastigote form may be converted to the axenic amastigote (a cultured extracellular form resembling the intracellular amastigote) by changing the culture conditions. Whole cell protein components of all three forms of Leishmania braziliensis panamensis, as well as samples taken during the promastigote to axenic amastigote conversion process, were analyzed by 2-D gel electrophoresis. While a number of proteins remained invariant, there were distinct differences in the overall protein patterns obtained from organisms at different stages of the conversion process and from the promastigote and intracellular amastigote forms. Studies are underway to correlate the changes in protein patterns with the infectivity of the organism.

5. Analysis of translation products from Leishmania donovani mRNA with various antisera. (In collaboration with the Dept. Immunolgy, DCD&I.)

Messenger RNA has been isolated from L. donovani promastigotes using guanidine hydrochloride precipitation followed by oligo (dT) cellulose chromatography. Poly (A) containing material was translated in a reticulocyte lysate system and the products analyzed by immunoprecipitation using: normal or patient human serum, monoclonal antibodies raised against whole promastigotes, normal and challenged mouse serum from two genetically different strains, and serum from mice which had received a second challenge with L. donovani after the initial parasitemia had abated. There were no apparent differences among the patient sera; nor were there any among the monoclonals. However, the mouse sera did show a pattern of appearance and disappearance of several protein bands of MW 20.000 - 40,000, (not appearing in normal mouse sera), the timing of which was different between the two strains. Upon rechallenge, one of the strains showed a reappearance of one of these bands within a much shorter time than seen in the original challenge. Messenger RNA isolated from *L. donovani* grown under two different growth conditions was analyzed by immunoprecipitation with mouse sera; there were no apparent differences. These studies are continuing through testing of new monoclonal antibodies and extension of the studies on culture conditions to correlate with data on surface antigen changes and infectivity.

6. Use of antisera and monoclonal antibodies to probe cDNA and genomic banks constructed in two dissimilar expression vector systems.

Monoclonal antibodies are being generated against purified membranes of *L. donovani*. Two host/vector expression systems have been chosen for cloning of genomic fragments. Random genomic fragments, prepared by specific restriction endonuclease treatment or most recently by non-specific (mung bean) nuclease treatment, used successfully for *Plasmodium* circumsporozoite protein (CSP), have been ligated into these vectors. Conditions for detecting cloned products using antiserum have been worked and are being used to screen existing clones for gene(s) of interest.

PROJECTED STUDIES

1. Continuation of studies on adenosine receptor binding in *Leishmania spp*. to include:

    a. adenosine binding to purified receptor proteins from isolated *Leishmania* membranes,

    b. the relationship between adenosine binding, membrane adenylate cyclase activity and c-AMP synthesis,

    c. the overall effect of adenosine binding on parasite proliferation and transformation and

    d. the effect of antileishmanial compounds on these systems

2. Continuation of studies on purine nucleoside transport and its association with 5' nucleotidase activity on the membrane of *Leishmania* promastigotes.

3. Use of available translation systems and two-dimensional gel electrophoresis to fully characterize products of messenger RNAs that are present during growth of promastigotes, correlating this data with infectivity and with changes in surface antigen composition as a function of time and conditions of culture.

4. Determine which cloned products correspond to molecules identified as important in 1 above. Subclone the coding fragments for sequence analysis. Utilize expression vector systems to produce enough antigen for animal immunoprotection tests.

5. Extend these studies to Leishmania spp. amastigotes. Identify antigens in common and determine the genomic repertoire for these antigens in both promastigotes and amastigotes.

PUBLICATIONS

1. Hansen, B.D., Webster, H.K., Hendricks, L.D. and Pappas, M.G. 1984. Leishmania mexicana: Purine metabolism in promastigotes, axenic amastigotes, and amastigotes derived from vero cells. Experimental Parasitology. 58, 101-109.

2. Hansen, B.D., Webster, H.K. and Wiesmann, W.P. 1984. The effect of mycophenolic acid on purine nucleotide metabolism of Leishmania mexicana mexicana. Molecular and Biochemical Parasitology. Accepted.

3. Jackson, P.R., Pappas, M.G. and Hansen, B.D. 1984. Fluorogenic substrate detection of viable intracellular and extracellular pathogenic protozoa. Science. In press.

ABSTRACTS

1. Hansen, B.D., Perez-Arbelo, J. and Chiang, P. K. 1984. Leishmania braziliensis panamensis (WR 120): Adenosine binding sites on the surface of the promastigote plasma membrane. American Society of Parasitiology, Supplement, Abstract 128.

2. Smejkal, R.M., Geller, R., Hansen, B.D. and Olenick, J.G. 1984. A two dimensional gel electrophoresis analysis of the conversion of Leishmania braziliensis panamensis promastigote to amastigote forms. Federation Proceedings. 43, 1791.

3. Jackson, P.R., Pappas, M.G., Jackson, J.E. and Hansen, B.D. 1984. Fluorogenic substrate detection of viable intracellular and extracellular pathogenic protozoa. Federation Proceedings. 43, 1250.

4. Jackson, P.R., Pappas, M.G. and Hansen, B.D. 1984. A rapid fluorescence microscopy procedure for determining the viability of intracellular and extracellular amastigotes and promastigotes of Leishmania. American Society of Tropical Medicine and Hygiene. Abstract 146.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION DA OG 9282	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636	

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY	61102A	3M161102BS10	CH	231	WWIG
b. CONTRIBUTING					
c. CONTRIBUTING	STOG 82/83-6.2/2				

11. TITLE (Precede with Security Classification Code)
(U) Studies of Military Personnel with Sickle Cell Trait (SCT)

12. SUBJECT AREAS
0603 Biology 0614 Personnel Selection and Maintenance

13. START DATE 82 01	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	4.0	239
85	5.6	293

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
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c. NAME OF RESPONSIBLE INDIVIDUAL
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20. PERFORMING ORGANIZATION
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f. NAME OF ASSOCIATE INVESTIGATOR (if available)
KARK, J A
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION H

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Sickle Cell; (U) Hypoxia; (U) Thalassemias; (U) Hemolysis; (U) RAM III

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23.(U) To determine medical risks to soldiers with sickle cell trait (SCT) who may be assigned to special military roles, such as Army aviation, which require performance during hypoxia. To perform studies of changes produced in their red cells by sickling to determine whether a laboratory test can be devised capable of predicting the relative susceptibility of SCT soldiers to complications of sickling as revealed in altitude chamber studies. These studies are important to Army aviation, because of its unique requirement to carry out missions in hypoxic environments, and because of a lack of data which define the incidence and degree of medical risk to individuals with SCT.

24.(U) A prospective, controlled study of the physiologic effects of hypoxia in an altitude chamber upon SCT aviator candidates and controls, using exposures similar to mission conditions and aviation training. Special procedures include chromatography of hemoglobins, D.I.C. and E.M. studies of red cells to identify sickle changes, use of autologous 51-Cr-RBC transfusion to measure splenic sequestration and hemolysis, use of lung scans and stage I exercise test to evaluate lung perfusion, overnight dehydration with AVP infusion to evaluate renal function, and measurement of endothelial proteins to detect endothelial damage.

8310 - 8409

25.(U) Hypobaric chamber studies were completed for 13 pairs of subjects. Initial analysis demonstrates reduction of lung perfusion for 5/13 men with SCT, but without effect on maximal exercise performance. Hypoxia had no effect on RBC survival or on splenic sequestration of RBC, although a separate effect of SCT on splenic capture of RBC has not been excluded. Data concerning in-vivo sickling, alpha-thalassemia, percent HbS, arterial SaO2, plasma Factor VIII levels, plasma prostaglandins, RBC density and size profiles, and urinary concentrating capacity are being analyzed. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project 3M161102BS10: RESEARCH ON MILITARY DISEASE, INJURY AND HEALTH HAZARDS

Work Unit 231 Studies of Military Personnel with Sickle Cell Trait (SCT)

Investigators COL John A. Kark, MC; LTC Daniel G. Wright, MC, MAJ James Canik, MC (AFIP); LTC David Posey, MC (AFIP); COL R.R. McMeekin (AFIP); CPT H.R. Schumacher (USN, AFIP) MAJ P.J. Tarassoff (WRAMC); MAJ J.A. Key (AFIP); Dr. H.L. Williams (GS-13)

Description

Individuals with sickle cell trait (SCT) may now enter previously restricted MOS which involve exposure to hypoxia and other stresses that can cause intravascular sickling in people with SCT and have been infrequently reported to cause medical complications.

1. Studies of altitude hypoxia in aviation.

The frequency and severity of complications in SCT members performing Army duties such as aviation, HALO parachute operations, and deep sea diving is unknown. It appears that aviation would provide the greatest risk since operational regulations allow one hour of flight at 10000 to 14000 ft in the ambient atmosphere and splenic infarction has been reported in at least 17 individuals with SCT under circumstances consistent with regulations. Nevertheless, the frequency and severity may be very low and the risk might be restricted to a few individuals with SCT and additional acute or inherited abnormalities. Better understanding of the pathophysiology of the sickling disorders is needed to devise lab tests capable of detecting the situations or individuals truly at risk. Currently DOD has requested removal of past restrictions upon SCT individuals from Army aviation, which would imply entry of SCT people into the hypoxic environment of aviation as the only responsible pilot for helicopters. It has not been known whether standard NATO hypobaric hypoxia training is safe for SCT individuals, nor what the risks would be for the longer episodes of hypoxia under more severe conditions during military operations. The Aeromedical staff of the Army have requested that we provide experimental data to establish the safety of the aviation environment in an orderly manner, so that SCT individuals may be brought into the role of helicopter pilot safely with minimum risk and with some factual basis for each successive level of risk which would be undertaken in their training and experience.

2. Studies of sudden life-threatening or fatal illness during basic combat training (BCT).

   Army duties also require rapid physical training of partially fit or unathletic individuals during BCT. Previous studies of case clusters suggest that recruits with SCT may have a high frequency of sudden collapse in BCT, followed by sudden death or by severe rhabdomyolysis. These studies did not define the relative risk for HbAA individuals and could represent recognition of random case clusters. Data on the relative risk and attributable risk of these complications, as well as a better case description are needed. If SCT carries an increased relative risk and a significant attributable risk, it will be necessary to define the pathophysiology of these catastrophic syndromes, to formulate appropriate preventive measures. A retrospective study, initiated in FY '82 was conducted and nearly completed in FY '84. A prospective study linked to the initiation of SCT screening for all Army recruits was planned.

Progress

1. Beginning on 15 April 1983 to 7 August 1984, 13 pairs of men with HbAA and HbAS were studied before, during, and after a series of hypobaric chamber flights to determine the effects of NATO altitude training upon men with SCT, with special attention to the potential for sickling and its complications. In addition to a thorough admission and discharge screening which included clinical evaluation, CBC, Hb electrophoresis, retic count, SMAC-20, CXR, and ECG, special tests where conducted to examine red cell turnover and splenic localization, CNS function (vision and psychomotor performance), the lungs (V/Q scans), the spleen (liver/spleen scans), the kidneys (maximal urinary concentration and urinary tissue marker proteins), cardio-pulmonary performance, and muscle performance (Stage I bicycle ergometer exercise tests). During hypoxia the chamber pressure, chamber P02, saturation of arterial blood with oxygen (Sa02), heart rate, stroke, volume, cardiac output, respiratory rate, and transthoracic impedance were continously recorded. Spot measurements were made of ventilation rate. end inspiratory gases, plasma free hemoglobin, venous percent sickled cells, and plasma endothelial marker enzymes. The effect of each flight on psychomotor performance, vision, retina, red cell

survival and red cell splenic localization (using 51-Cr labelling), biochemical markers of hemolysis, kidney concentrating ability, pulmonary function, retic count, CBC,

SMAC-20, and endothelial markers was tested. Lung ventilation (V) and perfusion (Q) scans were performed before and after the set of hypoxic exposures. The first ten pairs went through progressively greater hypoxia, with exposures to atmospheres equivalent to 5, 10, 14, 18, 20, 22, and 25,000 ft. The last 3 pairs underwent exposure to 25, 18, 14, 10, and 5,000 ft., since NATO training begins with exposure to the highest level of hypoxia, 25,000 ft. These individuals also received an additional lung perfusion scan injection, while at 14,000 ft. to test for the immediate effect of hypoxia on lung perfusion. The data has been only partially analyzed, but, surprisingly, there was no evidence for significant splenic sequestration of red cells. Equally surprisingly, 6 men with HbAS showed reduced lung perfusion on scans performed after or during hypoxia. This effect persisted for 4 to 8 days in 4 individuals who were re-studied. These results imply a greater effect of sickling on lung perfusion than expected from the previous literature and a smaller effect on splenic flow than expected from the previous literature. Taken together this data implies that the relative sparing of lung from sickle infarction, in comparison with spleen, is not, as previously thought, due to a difference in extent of vascular obstruction but may be mainly due to better maintenance of lung oxygenation during reduction of flow by sickling. These results also suggest that a closer examination of lung function is required. Both SaO2. stage I exercise testing and PFTs were in the normal clinical range for SCT men. Lung function was clinically adequate despite sickling and reduced perfusion. Nevertheless, the lung has considerable reserve capacity, so these studies do not rule out the possibility of significant loss of lung reserve function.

2. Studies of SCT in basic combat training

During FY '82-83, the AFIP PANLARS list of autopsies and the DMPDC list of basic trainees were combined to provide a tentative list of men who died in BCT and the numbers of Black and non-Black recruits in BCT for 1977-1981. Autopsy reports were obtained and reviewed for all individuals who died a natural death. These deaths were broken down into those with unexpected sudden collapse during BCT and other types of natural death. In FY '84, each BCT center was visited, the pathology reports reviewed and all cases potentially in BCT were recorded. Records were obtained and reveiwed for the autopsy reports on these cases, and deaths were catalogued, classified, and the relative risk of sudden unexpected fatal collapse or natural death for SCT and the attributable risks were calculated.

Plans were developed for a prospective study of recruits related to universal SCT screening, which would follow those positive for SCT and two control groups, racially matched and cross-over. Events followed would be those appearing in medical records, using life-table analysis. Lab evaluation would include associated risks, such as iron status, status of bone marrow and red cells evaluated by CBC, differential count, retic count, alkaline and acidic hemoglobin electrophoresis, %HbS, and G6PD screening.

3. Derivitive studies and consultations

(1) Studies have been completed which define the ability of pyridoxal phosphate to enter HbS containing red cells more rapidly than normal red cells, to react with HbS and to inhibit sickling by a mechanism different from pyridoxal (previously described by our laboratory). This agent has been shown to improve oxygen-transport in red cells, in contrast with most protein-modifying antisickling agents. This principal intracellular form of vitamin B_6 has been found to have very low toxicity in animal studies, as well as in its clinical use in humans. (2) Initial methods are being worked out in a human study "Anion permeablity of sickle cell trait red cells" (WRAMC #9021-82) and an animal study "Survival of pyridoxal-hemoglobin in the circulation" (WRAIR primate protocol #014-81) for the potential use of pyridoxal phosphate as an anti-sickling agent. (3) A case study of rare HbH disease in a Black Army man has led to the "Identification of a rare alpha-thalassemia-1 deletion." Also, a case study of an aviator with a rare Hb variant combined with HbS has led to the recognition that such a combination of variant HbS is equivalent to sickle cell trait for purposes of determining his participation in aviation. (4) A case study of an aviator candidate with SCT who had hemolysis during his Altitude Training indoctrination at the Brooke AFB hypobaric chamber revealed that this aviator had G6PD deficiency with sporadic mild hemolysis and no anemia. The flight surgeons at Brooke had attributed his hemolysis to SCT and were planning to exclude him from aviation. Our studies of 3 flights showed that no hemolysis was induced by altitude hypoxia, but that hemolysis occurred while at ground level. Pending confirmation from a reference lab that he has the common and benign "GdA-" variant of G6PD he may be able to obtain a

waiver to continue in aviation, since a waiver is usually granted for that condition.

Teaching was provided at the Aeromedical Operations Course, Ft. Rucker, AL. Consultation was provided to research groups at USARIEM, Natick, FAMC, CO, and WBMC, TX for studies of SCT.

Future Plans

1. A small survey of lung perfusion scans during and after ordinary NATO altitude training of SCT men will be conducted, to establish the incidence of these changes for the precise conditions used in training, since this involves exposure to two episodes of hypoxia, not studied as such in the Pilot study.

2. Continuation of the Pilot Study for operational length of exposure, with additional pulmonary and opthalmology studies, to include: multiple inert gas techniques to measure V/Q, and automated axial determinations of visual threshold in the chamber. In addition tests of experimental properties of red cells related to sickling which were planned for FY '84 could not be conducted because a cell physiologist was not available to perform these studies. A cell physiologist will join our lab in early FY '85 and these studies will be added.

3. Complete data analysis and interpretation of the Pilot Study and provide a plan for clinical monitoring of SCT aviator candidates during their NATO altitude training.

4. The retrospective survey of SCT individuals experiencing sudden unexplained death during basic combat training will be completed by review of clinical and administrative records at St. Louis to confirm the true BCT deaths for 1977-1981. There will also be an attempt to review spleen histology for iron overload suggestive of hemolytic disorders. SCT has been identified by hemoglobin electrophoresis, but absence of iron overload would support the differentiation from HbS/beta-thalassemia, which also gives a mixture of HbA and HbS on electrophoresis.

5. Survey of biophysical techniques which might be applied to a practical test to identify individuals with SCT at risk in military duties for sickling complications.

AVAILABLE

Abstracts

1. Embury SM, Monroy GC, and Kark JA. Identification of the rare alpha-thalassemia-1 deletion in the Black population. Blood 62 (Nov):66a, 1983.

2. Kark JA and Tarassoff PG. Pyridoxal Phosphate as an antisickling agent in vitro. Proceedings of the Workshop on extracorporeal therapy of Sickle Cell Disease. N.I.H., 1984.

3. Posey DM, Kark JA, McMeekin, and Schumacher HR. Sickle Cell trait associated with unexpected death in military basic trainees. Aviation, Space, and Environmental Medicine 55:459,1984.

4. Kark JA, van Nostrand D, Key JA, Tarassoff JJ, Canik JJ, Williams HL, Reba RC, Posey DM, Ruehle CJ, and Burge JR. Effects of altitude hypoxia on blood flow in the spleen and lungs of men with sickle cell trait. Blood, in press for Nov., 1984.

Published Articles

1. Winslow RM, Butler WM, Kark JA, Klein HG, and Moo-Penn W. The effect of blood letting on exercise performance in a subject with a high-affinity hemoglobin variant. Blood 62(Dec):1159-1164, 1983.

2. Kark JA, Haut MJ, Schechter GP, Reynolds, RD, Duffy TP, and Hicks CU. Changes in vitamin B6 metabolism in response to vitamin B6 in the sideroblastic anemias (in review), 1984.

3. Kark JA, Hannah JS, Goodman A, Agamanolis DP, Hines JD, and Harris JW. Altered central nervous system lipids in experimental vitamin B12 deficiency (in review), 1984.

4. Posey DM, Kark JA, McMeekin RR, and Schumacher HR. An association between sickle cell trait and fatal sudden unexpected collapse in military basic combat training (in review), 1984.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 300402	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AI	235 WWP3
b. CONTRIBUTING				
c. [illegible]	STOG 82/83-6.2/3			

11. TITLE *(Precede with Security Classification Code)*
(U) Ultrastructural Study and Definition of Diseases of Military Importance

12. SUBJECT AREAS
0603 Biology 0613 Microbiology

13. START DATE 81 10	14. ESTIMATED COMPLETION DATE Cont	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	5.0	268
85	5.0	230

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Pathology
b. ADDRESS *(include zip code)* Washington, D.C. 20307-5100	b. ADDRESS Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Baze, W B
d. TELEPHONE NUMBER *(include area code)* 202-576-3551	d. TELEPHONE NUMBER *(include area code)* 202-576-2307
21. GENERAL USE FINA MILITARY CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Hase, T g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Andersen, G L

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Host-Parasite Relationship; (U) Pathogenesis; (U) Ultrastructural Damage; (U) Repair; (U) Toxin; (U) Trauma.(U) Lab Animals; (U) Rabbits; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23(U) To study and define the ultrastructural bases of the pathogenesis of various injuries and diseases of military importance. Of particular interest are the structure and function of organs participating in oral immunity production, the nature of surface proteins of protozoans as related to vaccine development and structural changes in experimental animal models of nerve agent intoxication.

24(U) Conventional ultrastructural techniques, including transmission and scanning electron microscopy, as well as enzyme histochemistry, immunochemistry, negative staining, and autoradiography.

25(U) 83 10 - 84 09 Morphologic characteristics of gut immune organs were studied to help understand functional considerations which effect mucosal immunity. A technique developed to process small numbers of Trypanosomes for electron microscopy was utilized to help localize surface antigens. A new theory of rickettsial life cycle has been forwarded, based on morphologic studies. A protocol has been approved to study the ultrastructural aspects of Soman (nerve agent) intoxication in awake cats. Collaborative studies on the ultrastructural effects of microwave radiation on the eye were conducted. For technical report see the Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project 3M161102BS10 RESEARCH ON MILITARY DISEASES, INJURY AND HEALTH HAZARDS

Work Unit 235: Ultrastructural Study and Definition of Diseases of Military Importance

Investigators:
Principal: Wallace B. Baze, MAJ, VC
Assistants: Tatsuo Hase, MD
Douglas D. Sharpnack, CPT, VC
Eugene F. Bernard
Edward A. Asafoadjei

Description:

The primary purpose of this work unit is to define histopathologic manifestations of experimentally induced injury or disease which present current or potential problems to military personnel. Of particular interest are those aspects of injury or disease which are best studied at the ultrastructural level. A multidisciplinary approach, including conventional histology, histo-and cytochemistry, autoradiography, immunocytochemistry, and scanning and transmission electron microscopy, is employed.

This work unit also serves as a central electron microscopy facility for the WRAIR, offering services to include processing of tissues, electron microscope use (scanning and transmission), and photomicrograph processing. Various special techniques, such as negative staining and immunocytochemistry, are also available.

Progress:

I. Studies on Mucosal Immunity

The surface of gut associated lymphoid tissue (GALT) is exposed to a wide variety of antigens and microbes. The surface of various rabbit GALT, to include appendix, ileal Peyer's patches, sacculus rotundus and cecal lymphatic patch have been previously studied with all GALT examined containing lymphatic domes and some form of intervening villi or mucosal ridges. Studies this fiscal year and still in progress, include transmission and scanning electron microscopic study of: (1) developing GALT, (2) lymphoepithelial cells removed from rabbit appendices and (3) liposomes, delivered to the surfaces of Peyer's patches.

II. Studies on Tracheal Injury

This work unit has been investigating the cytodynamics of respiratory epithelial repair after mechanical injury in conjunction with ongoing WRAIR investigations on simulating the effect of repeated exposure to low levels of blast on operating various type of artillery weapons. Previous reports have described and quantified, at the light, transmission and scanning electron microscope levels, the morphologic and kinetic events that occur during regeneration following mechanical wounding of the hamster trachea. Epithelial sliding, squamous metaplasia, and ciliogenesis have been documented. Studies completed this fiscal year have shown similar morphologic and kinetic events in sheep and rats, following repeated blast exposure.

III. Studies the life cycle of *Rickettsia tsutsugamushi* in the Mammalian Cell

Rickettsial infections exist in certain regions of the world as endemic diseases. The potential for a massive outbreak of a rickettsial disease in a particular region is possible in a military operation where large numbers of unimmunized people enter the region under a stressful circumstance.

Members of the genus Rickettsiae are conceived as being obligatory intracellular bacteria; however, little is known about their life cycle with host cells.

We have previously accumulated morphologic evidence which suggested that rickettsia which appear within infected cells following inoculation may be assembled in site (1,2). In order to further clarify the replication made of the rickettsia, we have established the electron microscopic autoradiographic technique. This technique was applied to the study of the uptake of 3H-thymidine by irradiated L cells during the rickettsial infection. The results showed that 3H-thymidine supplemented in the medium was incorporated almost exclusively into the nuclei of infected cells, with little radioactive labeling occurring in intracytoplasmic rickettsia. Morever, the infected cells incorporated a much greater amount of the radioactive material into their nuclei than the control cells.

The significance of the increased radioactive incorporation into the infected cell nuclei is not clear at present, but, in view of the suggestive evidence of rickettsial assembly in host cells, the possibility that free rickettsial genomes replicate in the host cell nuclei must be considered (3). Further studies are planned to isolate free rickettsial genomes, to demonstrate their presence in the nuclei of infected cells and to rule out other alternative hypothesis for the autoradiographic findings.

The review article entitled "Developmental sequence and membrane affected by rickettsia" is in progress for publication in the Annual Review of Microbiology.

References:

1. Hase, T., 1983. Growth pattern of *Rickettsia tsutsugamushi* in irradiated L cells. J. Bacteriol. 154: 879-892.

2. Hase, T., 1983. Assembly of *Rickettsia tsutsugmushi* program in irradiated L cells. J. Bacteriol. 154: 976-979.

3. Hase T., and R.M. Rice. Autoradiographic study of 3H-thymidine uptake by *Rickettsia tsutsugmushi* infected irradiated L cells. J. Bacterial. In preparation.

IV. Central Electron Microscopy Facility

As a central electron microscopy facility for the WRAIR this department has either collaborated with and or supported several investigators, both within and outside the WRAIR. Collaborative studies have been conducted with: (1) COL Larsen of the WRAIR, on the ultrastructural effect of microwave radiation on the mouse eye with particular emphasis on the development of degenerative changes in the lens and (2) Dr. Siefer of Clinical Investigation, WRAMC, on the ultrastructural aspects of tendon repair in monkeys. In regard to support work, during this fiscal year, this facility received approximately thirty specimens per month for processing for transmission electron microscopy and approximately ten specimens per month for scanning electron microscopy. This is clearly a mission area with great potential for expansion, particularly as the in-house capabilities in immunoelectromicroscopy and subcellular antigen localization develops.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OG 2532
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AF	236 WRGS
b. CONTRIBUTING				
c. XXXXXXXXXX	STOG 82/83 -	6.2/3		

11. TITLE (Precede with Security Classification Code)
(U) Immune Mechanisms in Leishmaniasis

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE: 79 10
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	4.0	312
85	4.0	469

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
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(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME
Division CD&I
b. ADDRESS
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Hockmeyer, W T
d. TELEPHONE NUMBER (include area code)
(202)-576-3544
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
Nacy, C A
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Lab Animals; (U) Mice; (U) RAMI; (U) Immunity; (U) Leishmaniasis; (U) Tropical Medicine; (U) Macrophages

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objective is the elucidation of the mechanisms responsible for host destruction of leishmania during active disease or secondary challenge of immunized animals. This information will have a direct bearing on the feasibility of immunization against the disease and will provide methods for assessing immunity. Leishmaniasis extends throughout the tropics on every continent except Australia and is a threat to military operations. U.S. troops are contracting disease during training operations in Panama.

24. (U) The approach will be to identify immunomodulating agents that induce resistance to infection with Leishmania tropica in experimental animals.

25. (U) 83 10 - 84 09 BALB/c mice inoculated with L. tropica in footpads develop nonhealing cutaneous lesions followed by metastatic spread of parasites to the visceral. We investigate the possible protective effects of BCG treatment (a macrophage activating agent) on this lethal infection. Mice were inoculated ip with viable BCG or medium, infected in footpads with L. tropica, and then injected intra-lesionally with either medium, PPD, or viable BCG. All BALB/c mice inoculated with medium ip died of leishmania infection by 12 weeks. Of mice inoculated with BCG ip, those treated intra-lesionally with medium or PPD were not protected against the lethal disease (100 percent of medium-treated mice died, 70 percent of PPD treated mice died). However, BALB/c mice inoculated with BCG ip, then given viable BCG at the site of L. tropica infection were able to resolve the cutaneous lesion and did not develop systemic disease. Protected animals were completely immune to a second L. tropica challenge administered at a site remote from the primary infection. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

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PROJECT 3M161102BS10 RESEARCH ON MILITARY DISEASES,
INJURY AND HEALTH HAZARDS

Work Unit 236 Immune Mechanisms in Leishmaniasis

Investigators:

Principals:	Carol A. Nacy, Ph.D. LTC Wayne T. Hockmeyer, MSC
Associates:	MAJ David S. Finbloom, MC Beverly A. Mock, Ph.D. Anne H. Fortier

Problems and Objective:

The Leishmania are obligate intracellular protozoan parasites that replicate only within macrophages in mammalian hosts. The successful resolution of cutaneous lesions or systemic disease relies ultimately on the intracellular destruction of parasites by infected macrophages. The major interest of our laboratory is the documentation of parasite interactions with resident and inflammatory macrophages, and the analysis of changes induced in these interactions following nonspecific activation of macrophages by soluble lymphocyte products (lymphokines).

Progress:

To analyze parasite-host cell interactions *in vitro*, we developed a model assay with *Leishmania tropica* amastigotes and resident peritoneal macrophages of C3H/HeN mice. The parasite infects and replicates in these cells: infected macrophages maintained as nonadherent peritoneal cell cultures support 6 to 10-fold increases in the number of intracellular amastigotes over 72 hr (1). Addition of lymphokines to these cultures dramatically alters parasite-macrophage interactions, and lymphokine-treated macrophages develop two potent antileishmanial activities (2).

We now have evidence that lymphocytes can release soluble factor(s) that suppress macrophage intracellular destruction of *Leishmania* (3). PMA-stimulated EL-4 thymoma cell culture fluids were ineffective in activation of macrophages to kill the parasite; Sephadex G-100 fractionation of this same material, however, revealed a 50 kdalton peak of intracellular killing activity that induced 90-95% destruction of Leishmania in infected macrophages. Unfractionated EL-4 supernatants admixed with lymphokines, (LK) completely inhibited activation of macrophages for intracellular killing, but did not affect other activated macrophage effector activities, such as tumor cytotoxicity or resistance to infection. Preliminary characterization of this suppressor factor(s) showed: (a) suppression was dependent upon dose of EL4, (b) EL4 fluids added to LK-treated macrophages in the first 4 hr suppressed intracellular killing, but EL-4 fluids added after 4 hr of LK treatment were not suppressive, (c) in the 2-stage activation sequence, EL-4 fluids suppressed priming, but did not affect triggering of intracellular killing, and (d) EL-4 fluids had no effect on intracellular killing by macrophages activated as a consequence of BCG infection *in vivo*.

The EL4 suppressor factor(s) are nondialyzable, heat labile (45°C for 30 min; 56°C for 10 min), and relatively unstable during lyophilization (40-50% loss of activity). Analysis of gel filtration fractions suggests that this EL-4-induced suppression of antimicrobial activities may be mediated by more than one factor: (a) activity is not observed in individual fractions following separation on Sephadex G-100; (b) activity can be recovered by pooling fractions from 10-100K MW. Sequential pools of 5 consecutive fractions in the 10-100K MW regions were tested individually and in combination with every other pool: activity was only observed in those pools of 60-80K MW (4). Although immunosuppression is a documented sequella of leishmanial disease, the mechanism(s) by which suppressor lymphocytes exert their effects have yet to be defined. These T cell-derived soluble suppressor factors may provide insight into mechanisms of immunosuppression during leishmanial disease, and perhaps other intracellular parasitic infections.

BALB/c mice inoculated with *L. tropica* in footpads develop nonhealing cutaneous lesions followed by metastatic spread of parasites to the viscera; with inoculum doses of 1-5 million amastigotes, 90-100% of these mice die of systemic infection by 12 weeks. We investigated the possible protective effects of BCG treatment (a macrophage activating agent) on this lethal infection. Mice were inoculated intraperitoneally (ip) with viable BCG or medium, infected in footpads with *L. tropica*, and then injected intralesionally with either medium, purified protein derivative of tuberculin (PPD), or viable BCG. All BALB/c mice inoculated with medium ip died of leishmanial infection by 12 weeks. Of mice inoculated with BCG ip, those treated intralesionally with medium or PPD were not protected against the lethal disease (100% of medium-treated mice died, 70% of PPD treated mice died). However, BALB/c mice inoculated with BCG ip, then given viable BCG at the site of L. tropica infection were able to resolve the cutaneous lesion (footpad depths less than 1.5 mm), and did not develop systemic disease (no parasites in livers and spleens). These results suggest that protection from lethal leishmanial infections requires continuous activation of macrophages at the site of parasite inoculation. Of particular interest in this model of protection against leishmanial disease was the adjuvant effect of BCG-induced abrogation of the cutaneous lesion: protected animals were completely immune to a second L. tropica challenge administrated at a site remote from the primary infection (5).

Recommendations:

Problems associated with the control of leishmaniasis that will be investigated are: 1) evaluation of host-parasite interactions with soluble products of immune lymphocytes. 2) evaluation of nonspecific and specifically sensitized lymphocyte products for immunoprophylactic/therapeutic and diagnostic potential. 3) development of *in vitro* methods for assessing immunity, and 4) evaluation of nonspecific immunopotentiating agents for control of leishmanial infection.

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7. Fortier, A.H. 1984. Genetics of Leishmania tropica susceptibility in P/J mice. Catholic University of America, Washington, DC (May).

8. Mock, B.A. 1984. Genetics of resistance to Leishmania. Netherlands Cancer Institute, Amsterdam, the Netherlands (July).

9. Nacy, C.A. 1984. Genetics of L. tropica infections in the mouse, and In vitro analysis of macrophage activation for antimicrobial activities. Visiting Scientist, McGill University, Montreal, Canada (July).

10. Nacy, C.A. 1984. Characterization of a T cell factor that suppresses macrophage activation to kill intracellular Leishmania tropica amastigotes. Antibody and Lymphocyte Networks: Impact of Infectious Agents. Falls River, Vermont (August).

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1. Nacy, C.A., A.H. Fortier, and M.S. Meltzer. 1984. Macrophage activation to kill *Leishmania tropica*: characterization of P/J mouse macrophage defects for lymphokine-induced antimicrobial activities against *Leishmania tropica* amastigotes. J. Immunol. 133: 000 (in press, December).

2. Meltzer, M.S., D.L. Hoover, M.G. Gilbreath, C.R. Alving, G.M. Swartz, Jr. and C.A. Nacy. 1984. Macrophage activation for microbicidal activity against intra- and extracellular parasites. Proc. 10th Internat. RES Congress, Plenum Press (in press, November).

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4. Mock, B.A. and Gill, D.E. 1984. The impact of trypanosomes on ed-spotted newt survival and reproduction. Parasitology (in press).

5. D.S. Finbloom. Binding endocytosis, and degradation of mouse immunoglobulin G dimers and heavy oligomers by murine macrophages at various levels of activation. (Submitted for publication, J. Immunol.)

6. David S. Finbloom, Kristie Silver, David A. Newsome, Ralph Gunkel. The use of antimalarials and the development of toxic retinopathy. (Submitted for publication, J. Rheumatol.)

7. Gilbreath, M.J., C. A. Nacy, D.L. Hoover, C.R. Alving, G.M. Swartz, Jr., and M.S. Meltzer. Differential inhibition of macrophage microbicidal activity by liposomes. (Submitted for publication, Infection and Immunity.)

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1. Fortier, A.H., M.S. Meltzer, and C.A. Nacy. 1983. Genetic control of Leishmania tropica infection in P/J mice. J. Reticuloendothelial Society 34: 155 (October).

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PROJECT 3M161102BS11

CHEMICAL AGENT EFFECTS AND ANTIDOTES

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION DA 304954	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX
9. LEVEL OF SUM A. WORK UNIT					

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS11	EA	081 WWJR
b. CONTRIBUTING				
c.	STOG 82/83-6.2/2			

11. TITLE (Precede with Security Classification Code)
(U) Sequelae of Soman Exposure

12. SUBJECT AREAS
0601 Biochemistry 0616 Physiology 0615 Pharmacology

13. START DATE 83 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	0.0	28
c. TYPE	d. AMOUNT	85	0.0	34
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Division of Neuropsychiatry Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, Jr	c. NAME OF PRINCIPAL INVESTIGATOR Kant, G J
d. TELEPHONE NUMBER (include area code) 202 ,6-3551	d. TELEPHONE NUMBER (include area code) 202 -576-3302
21. GENERAL USE F I N A MILITARY CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Meyerhoff, J L
	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Walczak, D D

22. KEYWORDS (Precede EACH with Security Classification Code) (U)Chemical Defense; (U)Organophosphates; (U)Seizures; (U)Hypoxia; (U)Neuropeptides; (U)Amino Acids; (U)Neuronal Degeneration; (U)Lab Animals ;(U)Ra

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Determination of the effects of acute and long-term exposure to organophosphates on neurochemical, neuroendocrine, neuroanatomical and neurophysiological function in animals for the purpose of developing improved prevention and therapy for soldiers exposed to chemical warfare agents. This is of military relevance.

24. (U) Examination of brain neurotransmitter systems, both cholinergic and noncholinergic, neuropeptides, brain proteins and receptors, pituitary function and plasma hormones following acute or chronic organophosphate exposure. Examination of the response of these systems to challenge (e.g. stress) in organophosphate-exposed survivors at various times following exposure to assess return to normal function. Examination of the relationship between organophosphate-induced seizures and observed brain neuroanatomical damage following organophosphates.

25. (U) 83 10 - 84 09DFP was shown to inhibit the release of norepinephrine from cortical and hypothalamic tissue and dopamine from striatal tissue, thus demonstrating that this organophosphate can affect non-cholinergic neurotransmission as well as its known effects on cholinergic neurons. Two weeks following a single exposure to DFP (LD_{35}), rats displayed normal circadian rhythms for the plasma hormones: corticosterone, beta-endorphin, and beta-LPH. Prolactin levels were higher in DFP-treated rats at all time points tested. A pharmacological study of the effects of muscarinic and nicotinic anticholinergic drugs on seizure development was completed. A combination of atropine and mecamylamine (cholinergic blockers) was found to retard the development of seizures generated via the kindling model. For Technical Report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project 3M161102BS11 CHEMICAL AGENT EFFECTS AND ANTIDOTES

Work Unit 081 Sequelae of Soman Exposure
Investigators
Principal: Kant, G.J., Ph.D.
Associate: Meyerhoff, J.L., M.D., Walczak, D.D., Ph.D., CPT, Oleshansky, M.A., MAJ, M.D. Lynch, T.J., Ph.D., Mougey, E.H., M.S.

Objectives:

The objective of this research is to provide an improved data base regarding the effects of organophosphate (OP) and other chemical warfare agents on the functioning of the brain for the purpose of developing improved therapeutic measures for the prevention and treatment of chemical warfare casualties. The approach taken is to examine both acute and long-term sequelae of OP exposure on brain neurochemistry, neurophysiology and neuroanatomy. The effects of OPs on brain-regulated neuroendocrine function are also investigated.

OPs have been shown to cause seizures and also to result in neuronal degeneration. The interrelationship between these two OP effects is not well defined but is of critical importance in the design of antidotes to prevent long term sequelae of agent exposure. Research examining the relationships among OP exposure, seizure activity and central neuronal degeneration is ongoing.

Progress:

Although organophosphates are known to affect cholinergic neurons, the question remains as to whether OP's might also directly or indirectly affect brain non-cholinergic neurons. The effects of DFP on the release of neurotransmitters from minced brain tissue were measured using a very sensitive assay procedure that allows measurement of endogenous norepinephrine and dopamine release. DFP was shown to inhibit the release of norepinephrine from brain cortical and hypothalamic tissue and the release of dopamine from striatum. Thus, organophosphates might be expected to interfere with non-cholinergic neuronal systems as well as their better known cholinergic targets.

A number of studies were conducted using the putative cholinergic neurotoxin AF64A in order to better delineate the functions of cholinergic pathways in various behaviors, since the primary target of OP agents is the cholinergic nervous system. Two experiments demonstrated that AF64A could have non-specific effects on other neurotransmitter systems. When AF64A was injected into the lateral ventricles of rats, behavioral and neurochemical data were similar to those seen after a radiofrequency lesion of the fimbria-fornix, resulting primarily in damage to cholinergic neurons. However, when AF64A was injected into the substantia nigra, marked damage to dopaminergic cells ocurred. These data illustrate the need for careful histological and neurochemical data in AF64A-treated animals prior to ascribing observed behavioral effects of this drug to damage to cholinergic CNS. These studies were published during the year (see below) and have engendered considerable interest among scientists studying the cholinergic CNS.

Organophosphate exposure has been reported to cause long-term behavioral effects in humans and animals. Recent experiments in our Division have shown that a single injection of the organophosphate DFP disrupts normal circadian patterns of activity and eating for several weeks. Since cholinergic neurons (a prime DFP target) are involved in the regulation of endocrine function, we examined neuroendocrine parameters two weeks following a single injection of a LD_{35} of DFP. Since DFP disrupted behavioral rhythms, we examined hormones (which also display circadian rhythms) over a 24 hr period. The purpose of this experiment was to determine whether an OP disrupts normal hormonal rhythms at a time point when survivors have resumed eating normal amounts of food and appeared functional. Plasma hormones could be sampled in soldiers exposed to OP's and could serve a diagnostic function.

Three groups of animals were studied: controls, DFP-injected only and antidote-pretreated (atropine and 2-PAM) followed by DFP. As expected, antidote pretreatment greatly reduced the mortality rate as compared to DFP alone (2.5% vs 35%). Two weeks following a single treatment , groups of rats from each treatment group were sacrificed at 0600, 1000, 1400, 1800, 2200 and 0200 hrs. Following decapitation, trunk blood was collected and rectal temperatures immediately recorded (rectal temperatures were not recorded prior to sacrifice to avoid stressing the intact animal).

Body temperatures followed the usual circadian rhythm with a nadir at 1400 and the highest values recorded at 0200 to 0600 hrs. No significant differences were seen among treatment groups. Plasma Beta-endorphin levels followed the temperature pattern, with no significant differences observed among treatment groups. Peak values for corticosterone, prolactin and Beta-LPH were seen at 1800 hrs (the beginning of the rat activity period, lights off). The lowest values for corticosterone and prolactin were seen at 0600 hrs and the lowest Beta-LPH values were seen at 1000 hrs. There were no significant differences among treatment groups for corticosterone or Beta-LPH, while prolactin levels were highest in the DFP-treated group at all times tested.

A new seizure model has been developed for use in neurochemical and pharmacological studies of seizures. The Suprathreshold Stimulation Model

(STS) provides an easily-controlled acute seizure model that is intermediate in severity between maximal electroshock (MES) and pentylenetetrazol (PTZ) models. Like MES, the STS seizure is produced by electrical stimulation of the brain. Unlike MES, stimulation is applied directly to the cortex through permanent extradural electrodes, and the stimulation intensity is adjusted to elicit primarily clonic ('kindled-type') seizures. The STS model provides a 'seizure control' treatment equal in severity and duration to fully-generalized kindled seizures, and responds to anticonvulsants in a similar manner. We plan to utilize both the STS and MES seizure models as control treatments in studies of soman toxicity, to produce seizure experiences similar in severity and pattern to those expected to be observed in soman-treated rats.

A pharmacological study of the role of cholinergic function in the development of generalized seizures has been completed. The study tested the hypothesis that both muscarinic and nicotinic cholinergic function is involved in the progressive development of seizures in the kindling model of epilepsy. Rats were pretreated daily with either saline (SAL), the primarily muscarinic blocker atropine 20 mg/kg (ATRO), the nicotinic blocker mecamylamine 10 mg/kg (MECA), or a combination of both drugs in the same doses used previously (COMBO). Rats were then given electrical stimulation in the amygdala (1 sec train, 0.1 msec biphasic square pulses, 60 hz, 200 micro-amps) and their seizure activity was scored, both behaviorally and electrographically. Only the COMBO treatment was effective in attenuating seizure severity and increasing the number of stimulations required to elicit fully-generalized seizures. This treatment also shortened the duration of epileptiform activity in the EEG when generalized seizures finally did occur. These findings suggest that both muscarinic (Atropine-sensitive) and nicotinic (mecamylamine-sensitive) neuronal systems are involved in the expression and development of generalized seizures in kindling, and may be important targets for intervention in other seizure states.

A relatively new organophosphate poison was tested for its potential to cause brain hypoxia in rats during the epileptic seizures which it induces. The compound is the bicyclic organophosphate, ethyl phosphatrioxabicyclo octane (EPTBO), and it is the chief combustion product in the burning of fire retarded plastics. Unlike the standard organophosphate poisons, its primary neurophysiological effect is antagonism of the key inhibitory neurotransmitter, gamma aminobutyric acid (GABA). Rats were injected with an LD_{50} dose of EPTBO and the partial pressure of oxygen (pO_2) in hippocampal brain tissue was monitored polarographically during the seizures which followed. The average response during the onset of each seizure was a 32% decrease in brain pO_2, running its course in 40 seconds, followed by a pO_2 overshoot of 42%, running its course in 2 minutes. At this time we are uncertain whether this transient hypoxia is due to seizure-induced respiratory embarrassment or an increase in brain metabolism.

Future Objectives:

Continue to examine the effects of OPs on brain neurochemistry and neuroendocrine regulation. A planned collaborative study with USAMRICD will examine endocrine parameters in soman rat survivors. Assays for various neurotransmitter receptors are being setup by a NRC fellow. We plan to examine the effects of OP exposure on the status of brain neurotransmitter receptors and neuronal enzymes. The OPs used in future studies will depend upon the availability of agents and will be restricted to non-agent OPs unless a WRAIR agent facility becomes operational.

Continue to study the contribution of cholinergic systems to seizure development in kindling and other seizure models. Plan to perform continuous (24 hrs to several days) monitoring of EEG and seizure activity in Soman-treated rats pending the establishment of an accessible soman facility. These experiments will provide quantitative as well as qualitative data on the behavioral and electrographic effects of OP exposure in rats. Once this data is obtained, we plan to investigate the role of soman-induced seizures in acute and residual pathology associated with OP exposure, acute and longterm behavioral changes, and frank central nervous system pathology in OP exposure. We will utilize our recently developed electrical seizure models as non-OP 'seizure-experience controls'. These data may also be applied to testing of experimental antidote preparations.

Presentations:

1. POSTER presented at the annual meeting of the Federation of American Societies of Experimental Biology (FASEB), St. Louis, MO. in March of 1984. Title of Poster:
   Walczak, D.D. and Meyerhoff, J.L. Supra-Threshold Stimulation (STS): an electrically-induced seizure model proposed as a control treatment in neurochemical studies of Kindling.

Publications:

1. Jarrard, L.E., Kant, G.J., Meyerhoff, J.L. and Levy, A. Behavioral and neurochemical effects of intraventricular AF64A administration in rats. Pharmacol. Biochem. Behav. 21, 273-280, 1984.

2. Levy, A., Kant, G.J., Meyerhoff, J.L. and Jarrard, L.E. Noncholinergic neurotoxic effects of AF64A in the substantia nigra. Brain Research 305, 169-172, 1984.

3. Kant, G.J., Kenion, C.C. and Meyerhoff, J.L. Effects of DFP and other cholinergic agents on the release of catecholamines from brain regions *in vitro*. Biochemical Pharmacology 33, 1823-1825, 1984.

4. Landman-Roberts, L., Kant, G.J., Eggleston, T., Kenion, C.C., Driver, G.C. and Meyerhoff, J.L.. Atropine increases sensitivity to stress. Neuroscience Abstracts 9, 1123, 1983.

5. Walczak, D.D. and Meyerhoff, J.L. Supra-Threshold Stimulation (STS): an electrically-induced seizure model proposed as a control treatment in neurochemical studies of kindling. Federation Proceedings 43(3): 568 (1984).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA OC 6480	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS11	EB	219 WWH4
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2.1			

11. TITLE (Precede with Security Classification Code)
(U) Biochemical Aspects of Medical Defense Against Chemical Agents

12. SUBJECT AREAS
0601 Biochemistry 0603 Biology 0616 Physiology

13. START DATE 79 10	14. ESTIMATED COMPLETION DATE Cont	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	7.0	531
c. TYPE	d. AMOUNT			
e. KIND OF AWARD	f. CUM/TOTAL	85	2.0	271

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Biochemistry
b. ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Wolfe, A D
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-2312
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Doctor, B P g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Antidotes; (U) Acetylcholinesterase ; (U) Lab Animals; (Rats); (U) Guinea Pigs ; (U)RAMV; (U) Organophosphates

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objectives of this work unit are: (1) to provide the U.S. Army Command with a safe and effective prophylactic/therapeutic nerve agent antidote, (2) to develop additives which will convert stoichiometric nerve agent scavengers into catalytic antidotal formulations, (3) to determine the mechanisms by which formulation components protect against toxic neural agents, (4) to determine the interactions between components of antidotal formulations, (5) to identify optimal biological and chemical sources for antidotal preparation components, and (6) to determine the pharmacodynamics, enzymology, physiology, distribution, transport, metabolism, toxicology and immunology of such antidotal formulations.

24. (U) Macromolecules which combine with, or hydrolyze organophosphate threat agents will be purified in large quantity to test as therapeutic agents. Biochemical characteristics of purified macromolecules will be elucidated. Active center topography will be delineated by peptide mapping, amino acid composition and sequence determination, monoclonal antibody affinity, modulator influence and inhibitor affinity. Modulators which enhance enzyme activity will be studied, and optimal combinations of enzymes, reactivators and/or modulators will be determined for potential therapeutic applications. Animal model systems will be used to determine fundamental and optimal physiological and pharmacological parameters.

25. (U) 83 10 -84 09 Acetylcholinesterase from fetal bovine serum has been purified in large quantity. Selected structural studies have been carried out. It will be tested as a therapeutic agent for organophosphates. The amino acid sequence of trypsin digested active site containing peptide of Torpedo acetylcholinesterase has been determined. The distribution and clearance of aprophen show that it is rapidly cleared from the blood and it is distributed like a classical benzilate including crossing the blood-brain barrier. A pharmaceutical formulation has been developed in which oxime HI-6 is found to be fully stable for 18 months to date at room temperature. For technical report see WRAIR Annual Report 1 Oct 83 - 30 Sept 84.

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PROJECT: 3M161102BS11 CHEMICAL AGENT EFFECTS AND ANTIDOTES

WORK UNIT: 219 Biochemical Aspects of Medical Defense Against Chemical Agents

INVESTIGATORS:

Principal: Bhupendra P. Doctor, Ph.D.

Associates: Nesbitt D. Brown, M.S.; John G. Olenick, Ph.D.; Alan D. Wolfe, Ph.D.

Assistants: M. Judith Gemski, B.S.; R. Richard Gray, M.S.; Leo Kayzak, B.S.; Robert Rush, Ph.D.; SP4 Margaret G. Stermer-Cox; SP4 Jeff S. Verdier; John S. Ralston, Ph.D.

DESCRIPTION:

The objective of this work unit is to conduct multifaceted biochemical research on chemical agents in order to provide the military with a safe and effective prophylactic/therapeutic formulation against chemical agents. These include: the identification of the metabolites and degradation products of chemical agents and antidotes; the determination of the pharmacodynamics, distribution, transport and metabolism of chemical and antidotal agents; the investigation of the effects of chemical agents and antidotes on enzyme catalysis, specifically on active sites of acetylcholinesterases and butyrylcholinesterase. The following investigations were conducted:

a. The metabolic disposition and metabolism of aprophen has been studied in rats.

b. The stability of aqueous solutions if HI-6 has been studied as functions of pH, temperature and additives.

c. The separation of HI-6 and its degradation products by ion-pair HPLC.

d. The separation and identification of a plasma and urinary monoacetylated conjugate of chloroquine in man by ion-pair HPLC.

e. Fetal bovine serum acetylcholinesterase has been purified and characterized.

f. Aprophen has been studied as both an inhibitor of and substrate for serine hydrolases.

g. Ornithine decarboxylase inhibition in plasmodia-infected red cell.

h. The perturbation of polyamine metabolism in human malarial cultures by the antimalarial drug, qinghaosu.

1. Elucidation of Aprophen Pharmacodynamics.

Elucidation of aprophen mammalian pharmacodynamics is required to determine the mechanism of action of this drug. Difficulty has been encountered in recognition of the technical parameters which clearly define necessary experiments. However, we have recently perfected a surgical procedure which reveals the fate of aprophen in rats. In these studies, four animals were injected with 20 μC_i of (^{14}C) aprophen, and blood samples were removed at 1, 5, 10, 20, 30, 60 and 120 minutes post injection. Samples were treated to remove red blood cells, and the plasma was extracted with ether to remove aprophen and its metabolites. Tissue samples of adrenal glands, brain, heart, kidney, liver, lung, muscle and spleen were also removed and processed with ether for chemical analysis. Results show that aprophen blood clearance is very rapid. Five minute blood samples contained at most 10% of the (^{14}C) aprophen present in 1 minute. Subsequent aprophen blood clearance was very slow. Concurrently, ten radioactive peaks appeared in these samples. Tissue samples, similarly assayed by HPLC techniques, showed the spleen to contain the largest number of peaks, and 48% of the total radioactivity. The lungs contained 27% of the (^{14}C) aprophen products, while the liver and kidney also contained substantial amounts of radioactivity. We have not yet identified any of the compounds present in these samples. Mass spectrometry is currently being utilized for this purpose.

2. Stability of HI-6.

Previous studies carried out in these laboratories showed that the stability of HI-6 dichloride in aqueous solutions is pH and temperature dependent. Current studies show that the chemical parameters established earlier are applicable for stabilization of HI-6 at various concentrations when the guidelines are followed. Simple and mixed aqueous preparations of HI-6 were routinely analyzed to determine their stabilities during storage periods up to twenty months. Using experimental parameters, where samples were stored at ambient temperature (18°C-20°C) and at incubated temperatures (40°C), HI-6 in citric acid/sodium citrate, acetic acid/sodium citrate, acetic acid/sodium acetate and in dilute hydrochloric acid was evaluated for its reaction kinetics. The pH 4.0 preparations were sealed in glass ampules for the designated time periods until assayed. Biweekly analyses of the samples were performed using ion-pair reverse-phase chromatographic systems. Spectrophotometric analyses were also performed to observe and compare differences in the resulting profiles. Results from this study show that HI-6 solutions of all formulations containing 12.5 mg/ml were stable at all temperatures where 25°C was not exceeded. The 40°C samples showed degradation of 90% of its original content after six weeks. We also observed that when concentrations of HI-6 were 250 mg/ml or more, stability of the HI-6 was reduced. However, if samples of this concentration were maintained at 4°C, stability can be maintained for extended time periods. Studies are continuing on the mechanisms involved in this degradation process.

3. Separation of HI-6 [4-Carbamyl-2'-hydroxyiminomethyl-1, 1'-Oxydimethylen-di (Pyridinium Chloride)] and its degradation Products By Ion-Pair HPLC.

Guidelines were established for characterizing HI-6 and its degradation products at various pH and temperature conditions. The study showed that the stability of HI-6 was pH dependent, as well as temperature and concentration dependent. From these studies, it was shown by HPLC that where pH and temperature were allowed to exceed established guidelines, 85% of HI-6 was degraded to at least five major products. Possibily, as many as thirteen peaks were observed. Using physicochemical techniques, including mass spectrometry, these degradation products are being characterized. The effect of long term storage on stability of HI-6 in aqueous solutions is being investigated at the present time.

4. Separation and Identification of a plasma and urinary monoacetylated conjugate of chloroquine in Man by Ion-pair HPLC.

The separation and identification of an new acetylated conjugate of chloroquine has given insight to metabolism of this antimalarial drug in humans. Prior studies pertaining to the acetylation of certain therapeutic drugs, especially those containing primary and secondary amino groups, showed that this process does occur in man. However, chloroquine has never been shown to be an N-acetyl-metabolite in humans during this pharmacokinetic process. This process of acetylation may be applicable for assessing the genetic polymorphism of an individual, thereby defining the state of well-being in human subjects.

5. Fetal Bovine Serum Acetylcholinesterase.

Acetylcholinesterase [(AChE) E.C. 3.1.1.7] from fetal bovine serum (FBS) was purified to electrophoretic homogeneiety. The procedure involved procainamide affinity chromatography with native FBS, followed by chromatography on Separose 6B and DEAE-Sephadex. The AChE was purified approximately 44,000 fold and 13 mg was obtained with relative ease. Substitution of batch affinity gel procedures has indicated that much larger volumes of FBS may be processed to yield much higher quantities of enzyme.

The purified enzyme was stable at 4°C for at least eight weeks, but was labile to freezing. FBS AChE exhibited typical substrate inhibition, had a K_m of approximately 120 μM and a turnover number of 5300 sec^{-1} with the substrate acetylthiocholine. FBS AChE was highly sensitive to the specific inhibitor BW284C51. The enzyme has a Stoke's radius of 76A as judged by gel filtration and from this a molecular weight of 340,000 daltons was calculated. FBS AChE had a subunit weight of 83,000 daltons by SDS gel electrophoresis; paraoxon titration indicated a relative active site mass of 75,000 daltons. FBS AChE therefore appeared to be a tetramer. The amino acid composition of FBS AChE was generally similar to those of both mammalian and electric fish AChEs; however, the FBS AChE contained less lysine than these enzymes. FBS AChE failed to bind triton

X-100. The lower lysine concentration, lectin affinity and failure to bind triton X-100 are characteristics which serve to distinguish FBS AChE from electric fish, bovine erythrocyte, and other AChEs. Monoclonal antobodies to FBS AChE have been raised, and are under investigation. The human erythrocyte monoclonal antibody AE-2 bound avidly to FBS AChE, suggesting epitope conservation.

6. Interactions between Aprophen and Serine Hydrolases.

Aprophen is a potent reversible inhibitor and a poor substrate of human serum butyrylcholinesterase (BuChE). Complex, mixed, competitive non-competitive inhibition kinetics were observed; an apparent competitive inhibition constant was estimated to be 3.7×10^{-7} M. BuChE hydrolysis of aprophen to diphenylpropionic acid and diethylaminoethanol did not appear to follow Michaelis-Menton kinetics. The BuChE turnover number for aprophen is 2.0×10^{-3} sec^{-1}. Rabbit liver oligomeric and monomeric carboxylesterases (CE) also hydrolyze aprophen with a similar turnover number that varied between 1.4×10^{-3} sec^{-1} to 4.3×10^{-4} sec^{-1}, respectively. Comparison of the catalytic rate of aprophen hydrolysis with butyrylthiocholine (BTC) and the neutral aromatic substrate, phenylthiobutyrate ØTB, indicated that BuChE hydrolyzes BTC and ØTB 3.2×10^{5} and 3.1×10^{5} times more rapidly than aprophen. Similarly, the CEs also hydrolyze BTC and ØTB 17.6 and 1.9×10^{5} times more rapidly than aprophen. Acetylcholinesterases from bovine erythrocyte and electric eel failed to be inhibited by aprophen, nor was aprophen hydrolyzed by these enzymes. The hydrolysis and inhibition reactions may best be described by a complex reaction scheme involving multiple binding sites for both the substrate and the inhibitor as well as positive cooperative ligand binding. Results suggest that *in vivo*, aprophen may be hydrolyzed by many different serine hydrolases and, in turn, may inhibit many of these enzymes. It should be noted that enzymes hydrolyzing aprophen, or which are inhibited by aprophen, are serine hydrolases sensitive to organophosphate threat agents. It is reasonable to infer that aprophen, to a limited extent, may protect serine hydrolases to which it binds.

7. Ornithine Decarboxylase Inhibition and the Malaria Infected Red Cell: Model for Polyamine Metabolism and growth.

The addition of D, L-α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, to human *Palasmodium falciparum*-infected red cells in continuous culture decreased both parasite growth and intracellular polyamine concentrations. Growth of *P. falciparum* in infected red cells was assessed by parasite counts and by assays for macromolecular syntheses of protein, DNA and RNA. Polyamine concentrations were measured by an automated high-performance liquid chromatography method. Concentrations of D, L-α-difluoromethylornithine > 0.3 mM decreased intracellular levels of putrescine, spermidine and spermine, reduced ornithine decarboxylase activity and the growth and maturation of the intracellular parasite at the trophozoite stage. There was a concomitant decrease in the synthesis of protein and nucleic acids in the infected cells. The use of this parasitized red cell model system together with polyamine inhibitors provide means of studying polyamine synthesis and cell growth in the design of new antimalarial therapy.

8. Qinghaosu, A Potent Antimalarial, Perturbs Polyamine Metabolism in Human Malaria Cultures.

Qinghaosu or Artemisinine, a natural product of the herb, qinghao or *Artemisia annua* has been shown to be an effective antimalarial agent against the parasite, *Plasmodium falciparum*. This sesquiterpene lactone compound, first characterized by scientists of the People's Republic of China, has a unique configuration that makes it different from most antimalarial compounds now in use. Qinghaosu has potent inhibitory activity against the pyrimethamine resistant parasite, Camp/Malay strain, with an ED_{50} of 6.8×10^{-9}M. Polyamine levels of cell pellets from parasitized RBC showed drastic changes in their content when compared to untreated normal RBC. Putrescine levels fell more than 66% in the treated cell pellets. At the same time, increasing levels of spermidine and spermine were observed in Qinghaosu treated cells. In contrast, spermine levels were more dose-dependent than those of putrescine and spermidine. From these evaluations, further studies are planned to examine the mode of action of this drug on polyamine biosynthesis pathways.

PROJECTED STUDIES

1. Purification and characterization of serine hydrolases will continue.
2. Structural and kinetic studies on serine hydrolases will continue.
3. Cholinesterases will continue to be mapped by monoclonal antibodies and HPLC analysis of tryptic digests.
4. Serine hydrolase protection of animals from organophosphate intoxication will be evaluated.

PUBLICATIONS

MANUSCRIPTS:

1. Brown, N.D., Gray, R.R., Stermer-Cox, M.G., Doctor, B.P. and Hagedorn, J., Stability study of HI-6 dichloride in various formulations as determined by ion-pair high performance liquid chromatographic and spectrophotometric analyses. 1984. J. Chromatogr., in press.

2. Brown, N.D., Stermer-Cox, M.G., Doctor, B.P. and Hagedorn, J., Separation of HI-6 [4-carbamyl-2'-hydroxyiminomethyl-1, 1'-oxydimethylen-di (pyridinium chloride)] and its degradation products by ion pair high-performance liquid chromatography. 1984. J. Chromatogr. 292, 444.

3. Brown, N.D., Stermer-Cox, M.G. and Poon, B.P., Separation and identification of a plasma and urinary mono-acetylated conjugate of chlorquine in man by ion-pair high-performance liquid chromatography. 1984. J. Chromatogr. 309, 426.

4. Rush, R.S., Ralston, J.S. and Wolfe, A.D., Aprophen: a substrate and inhibitor of human butyrycholinesterase. 1984. Biochem. Pharm., in press.

5. Ralston, J.S., Rush, R.S., Doctor, B.P. and Wolfe, A.D., Acetylcholinesterase from fetal bovine serum. Purification and characterization of a soluble G4 enzyme. 1984. Submitted to J. Biol. Chem.

6. Whaun, J.M. and Brown, N.D. Ornithine decarboxylase inhibition and the malaria infected red cell: A model for polyamine metabolism and growth. 1984. J. Pharm. and Exp. Therap., in press.

7. Whaun, J.M., Brown, N.D., Milhous, W., Lambros, C. Scovill, J., Lin, A. and Klayman, D. 1984: Qinghaosu, a potent antimalarial, perturbs polyamine metabolism in human malaria cultures. In polyamines: Basic and Clinical Aspects, ed. O. Suzuki, (Satellite Symposium of the Third International Congress on Cell Biology), VNU Science Press, Netherlands, in press.

8. Wolfe, A.D. Mefloquine. 1983. Antibiotics VI: 108 (Springer-Verlag, Berlin, Heidelberg, New York, Tokyo).

ABSTRACTS:

1. Ralston, J.S., Rush, R.S., Wolfe, A.D. and Doctor, B.P., Purification and characterization of acetylcholinesterase from fetal calf serum. 1984. Fed. Proc. 43 (7): 2007.

2. Rush, R.S., Ralston, J.S., Verdier, J.S., Doctor, B.P., Chiang, P.K. and Wolfe A.D., Hydrolysis of aprophen by cholinesterases and carboxylesterases. 1984. Fed. Proc. 43(6): 561.

3. Rush, R.S., Ralston, J.S., Wolfe, A.D., Marasco, J.M. and Doctor, B.P. Characterization of purified fetal calf serum acetylchlinesterase. 1984. Fed. Proc. 43(7): 2007.

4. Whaun, J.M. and Brown, N.D., A new ornithine analogue with antimalarial activity. 1984. 37th Annual Meeting of the Society of Protozoologists. Athens, Georgia.

5. Whaun, J., Brown, N.D., Milhous, W. Lambros, C. and Klayman, D. 1984. Qinghaosu, a potent antimalarial agent, perturbs polyamine metabolism in human malaria cultures. Polyamines: Basic and Clinical Aspects, a Satellite Symposium of the Third International Congress on Cell Biology, Gifu, Japan.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OG 6753	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS11	EA	221 WWJ4
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 82/83-6.2/1			

11. TITLE (Precede with Security Classification Code)
(U) Neural Mechanisms of Chemical Defense-Related Compounds

12. SUBJECT AREAS
0601 Biochemistry 0615 Pharmacology 0616 Physiology

13. START DATE 80 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	387
85	2.0	196

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Neuropsychiatry
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Campbell, C B G
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-3067
21. GENERAL USE F I N A MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Petras, J M g. NAME OF ASSOCIATE INVESTIGATOR (if available) Mobley, W C

22. KEYWORDS (Precede EACH with Security Classification Code) (U)Chemical Defense;(U)Chemical Interactions;(U)Sensory Motor Processing;(U)Experimental Neuropathology;(U)Rats;(U)Cats;(U)Monkeys(U)RAMV

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Investigations are directed at understanding the effects of chemical defense-related compounds on nervous system function. These studies have both direct and indirect military relevance.

24. (U) Animal experiments are based on anatomic methods for locating critical sites of agent/antidote action; on pharmacologic and biochemical methods for elucidating interactions between agents/antidotes and the body's chemistry; and on physiologic methods for studying the effects of agents/antidotes on neural signal processing.

25. (U) 83 10 - 84 09. macaque monkey brain tissue from animals exposed to soman that had experienced seizures and developed brain infarcts were studied further. This was done to determine whether or not the failure to find brain cell degeneration as seen in similarly exposed rats and cats, and would be expected from brain infarcts of whatever cause, might not be due to inadequacies in the staining procedure. The parameters of the staining methods were varied and tissue from a macaque subjected to a mechanically produced brain lesion were stained along with the other tissue. Although the control monkey material consistently showed neuron degeneration, that from the soman-exposed monkeys did not. The most likely explanation of these results is that the post-operative survival times of the soman-exposed monkeys was not optional and that these studies should be repeated with this in mind. Segments of the intercostal, phrenic, and sciatic nerves were examined under the electron miroscope for evidence of peripheral neuropathy. None was found. Rats exposed to phospho-trioxa-bicyclo-octane (PTBO), a cyclic organophosphate, showed no brain damage other than that found in nonexposed controls, where its presence could be explained on the basis of viral infection. The response of neonatal rat central cholinergic neurons to nerve growth factor was studied. Changes in choline acetyltransferase activity were used as a marker. For Technical Report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 1498 83 MAR   EDITION OF MAR 68 IS OBSOLETE.

Project: 3M161102BS11 Chemical Agent Effects and Antidotes

Work Unit: 221 Neural Mechanisms of Chemical Defense Related Compounds

Investigators:
Principal: C.B.G. Campbell, M.D., Ph.D., LTC, MC
Associate: J.M. Petras, Ph.D., W.C. Mobley, M.D., Ph.D., MAJ, MC

Objectives:

The overall objectives of this task are the protection of military personnel from the lethal effects of chemical agents, elimination or reduction of impaired performance , and return to duty of those individuals exposed to sublethal doses of these agents. Emphasis has been placed on determining the sites of action of chemical defense-related compounds, the primary and secondary effects of these agents and their antidotes on nervous tissue, and exploring the possibility of exploiting the intrinsic defense mechanisms of the body as protection against these agents.

Progress:

Brain tissue from two Rhesus monkeys that had experienced seizures following soman intoxication have been studied further. Both animals had bilateral infarcts in the basal ganglia, internal capsule, and substantia nigra. Axonal degeneration as demonstrated by the Nanta-Gygax procedure and its various modifications was not found. This contrasts with positive findings in rats and cats. Technical difficulties with the soman injection, postoperative survival time, the staining procedure, or a combination of these factors was suspected. Axonal degeneration usually results from brain infarcts of whatever cause. During this fiscal year considerable time and effort was expended in varying the parameters of the staining methods. Further, a deliberate lesion was placed in the cerebral hemisphere of a control monkey. Tissues from the brain of this monkey were processed along with those of the brains containing infarcts. Although the control material demonstrated axonal degeneration readily, that from the soman-exposed monkeys did not. These results leave unresolved the question as to whether or not soman exposure can produce in primates the same kind of brain damage seen with this agent in rats and cats. It appears most likely that the failure to demonstrate axonal degeneration in monkeys thus far is a technical matter. For example, the post-exposure survival time may not be optimal. The study needs to be repeated.

Segments of the intercostal, phrenic, and sciatic nerves from the two monkeys with brain infarcts following soman exposure were examined under the electron microscope for evidence of peripheral neuropathy. No abnormalities were seen. Survival times after intoxication were not as long as usually used to demonstrate peripheral neuropathy with non-agent organophosphates. This study bears repeating with survival times appropriate to the development of peripheral neuropathy. Rats exposed to phospho-trioxa-bicyclo-octane (PTBO), a cyclic organophosphate, in a dosage range of 0.12 - 1.1 mg/kg were examined for evidence of neuronal degeneration in survivors. Degeneration was confined to the cerebellum and central acoustic system, sites of degeneration also found in non-exposed controls. The neuron damage seen in this instance may be a result of viral infection in the colony. Immunocytochemical protocols have been developed which make it possible to visualize cholinergic nerve terminal networks in cat and monkey brains. Abnormal cholinergic neurite terminations have been observed in the brains of aged monkeys. Some of these are found in association with amyloid in the neurite plaques of these animals. These data have provided further evidence of cholinergic system involvement in the pathogenesis of plaques. The high degree of correlation between plaque density and dementia scores in patients with Alzheimer's disease suggests that these neuritic abnormalities may be tied to the intellectual decline of these patients. It is possible that in survivors similar abnormalities could be produced by organophosphate nerve agents which attack cholinergic neurons. If such is the case, then plaque formation may be used as a morphologic indicator of cholinergic damage in survivors of organophosphate exposure in animals. This might provide a means of grading the degree of damage and the effectiveness of pretreatments or therapies directed against nerve agent intoxication.

Recommendations for Future Work:

If, as currently planned, facilities for intoxication of experimental animals with nerve agents are available at the WRAIR and additional rhesus monkeys are available for experimentation, the soman exposure study should be repeated using varying survival times. Investigations with sarin exposure in rats, looking for evidence of brain damage in survivors should be performed. Abnormal cholinergic nerve terminals should be sought in survivors using the immunocytochemical techniques mentioned above. The possible role of nerve growth factor (NGF) in defense against anticholinergic nerve agents should be pursued.

PUBLICATIONS

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Kitt, C., Price, D., Struble, R., Cork, L., Wainer, B., Becher, M., and Mobley, W. (1984) Evidence for cholinergic neurites in senile plaques (accepted for publication in Science).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA OH 0383	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS11	EB	227 WWIF
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/1			

11. TITLE (Precede with Security Classification Code)

(U) Chronic Systemic Effects of Organophosphate Esters

12. SUBJECT AREAS

0615 Pharmacology 0616 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
82 05	CONT	DA	C. In-house

17. CONTRACT GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	2.5	205
c. TYPE	d. AMOUNT			
e. KIND OF AWARD	f. CUM/TOTAL	85	2.5	210

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Division of Medicine Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR SMALLRIDGE, R C
d. TELEPHONE NUMBER (include area code) (202)576-3551	d. TELEPHONE NUMBER (include area code) (202)576-3014
21. GENERAL USE FINA	f. NAME OF ASSOCIATE INVESTIGATOR (if available) WHORTON, N E
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available) FEIN, H G

22. KEYWORDS (Precede EACH with Security Classification Code) (U)Pituitary hormones;(U)Lab animals;(U)Dogs;(U)Rats;(U)Organophosphates;(U)Anticholinesterase;(U)Cardiac enzymes;(U)Lung receptors;(U)Ram V

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23.(U) To determine the systemic effects of low dose single or repeated exposure to organophosphates. These studies will examine the effect of such agents and other cholinergic drugs on heart and lung enzymes and receptors, and on secretion of the anterior pituitary hormones. This project has military relevance by exploring mechanisms by which nerve agents may produce alterations in function of the heart, lungs, and pituitary gland.

24.(U) Animal models will be used to produce sublethal injury with single or repeated dose exposures. Cardiac and pulmonary effects will be studied by examining selected enzymes and receptors using cholinergic angonists/antagonists in vitro and in vivo. Pituitary hormone secretion in response to these agents will be measured both in vivo and in vitro using a dispersed cell pituitary perifusion system.

25.(U) 8310 - 8409 Lung angiotensin-converting enzyme (ACE) activity was recently shown to be inhibited in vitro by physostigmine, an effect reversed by atropine. Studies are in progress testing the effect of in vivo atropine administration on rat lung and serum ACE and on serum pituitary hormones. Cholinergic regulation of thyrotropin and growth hormone secretion were demonstrated in a human pituitary tumor grown in cell culture. Short term cultures of rat pituitary cells have been established, and a perifusion system is nearing completion. This model will be used to study in vitro the pulsatile release of pituitary hormones, and their interaction with cholinergic drugs. Serum samples from dogs receiving nonlethal doses of DFP are currently being analyzed for the effect of this organophosphate on pituitary hormone levels. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

Project: 3M161102BS11 CHEMICAL AGENT EFFECTS AND ANTIDOTES

Work Unit 227: Chronic Systemic Effects of Organophosphate Esters

Investigators:
Principal: Robert C. Smallridge, LTC(P), MC
Associate: Nancy E. Butkus, GS-11
Henry G. Fein, MAJ, MC
Irene Gist, SP4
Thomas J. Wheeler, SP4

This work unit was established to study the chronic systemic effects of organophosphate esters in a rodent model and on selected organ systems in vitro. The acetylcholinesterase to be used is diisopropylfluorophosphate (DFP). Studies are being performed in three areas.

1. Cardiac Studies: Organophosphates can reduce cardiac output and contractility (1,2). The mechanism, though unknown, may be via ATPase (3,4). Studies in this area are to include (a) analysis of several myocardial enzyme activities and (b) hormonal receptors after in vitro or in vivo exposure to cholinergic drugs and their antagonist (atropine). The loss of the remaining biochemist in the department prevented any work this year.

2. Pulmonary Studies: Respiratory depression is the usual cause of death after acute exposure to nerve agents. Chronic effects on pulmonary function are unknown. Thyroid hormone promotes surfactant production (5), and lung receptors for this hormone have been partially characterized. Work in this area has been temporarily inhibited because the investigator has been involved extensively in studies of atropine pharmacokinetics this year.

3. Hormone/Metabolic Studies: The anterior pituitary gland has cholinergic receptors, and several of its hormones are under cholinergic control (6-13). Considerable progress has been made in this area. Two animal models (rat and dog) are being used. Radioimmunoassays for many of the pituitary hormones in both species have been established and validated this year. Sera have been collected from dogs exposed to DFP, and are currently being analyzed to see if acute exposure to this agent affects circulating pituitary hormone levels.

A laboratory has been established for performing cell cultures, and short-term pituitary cultures are underway. These cells are used in a perifusion system (14) (recently established and validated in this laboratory), and will permit the in vitro study of cholinergic modulation of hormone secretion both basally and in response to hypothalamic releasing hormones and neurotransmitters. A human pituitary tumor secreting both growth hormone and thyrotropin was cultured. Cholinergic control of both hormones was documented (15).

Angiotensin-converting enzyme (ACE) has been under study for several years. This enzyme is important in blood pressure regulation because of its interaction in the renin-angiotensin system. Last year, *in vitro* studies indicated cholinergic inhibition of the enzyme with physostigmine, and reversal with atropine. Further studies have been done in humans in critical illness. In rats, ACE activity is currently being measured in serum, lung, and kidney after atropine injection. Serum atropine levels are also being monitored.

Future research plans include: (a) studying *in vitro* and *in vivo* the effects of cholinergic agents on secretion of pituitary hormones, (b) continuation of projects involving regulation of ACE activity, and (c) resumption of receptor and cardiac enzyme studies.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA OH0169	84 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D Change	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS11	EC	232 WWSF
b. CONTRIBUTING				WWGT
c. CONTRIBUTING	STOG 82/83-6.2/1			

11. TITLE (Precede with Security Classification Code)

(U) Immunochemistry of Nerv. Agents

12. SUBJECT AREAS

0620 Toxicology 0615 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
82 04	Cont	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE

EXPIRATION

b. CONTRACT/GRANT NUMBER

c. TYPE

d. AMOUNT

e. KIND OF AWARD

f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	1.0	149
85	1.0	159

19. RESPONSIBLE DOD ORGANIZATION

a. NAME

Walter Reed Army Institute of Research

b. ADDRESS (include zip code)

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c. NAME OF RESPONSIBLE INDIVIDUAL

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20. PERFORMING ORGANIZATION

a. NAME

Division of CD&I

b. ADDRESS

Walter Reed Army Institute of Research
Washington, DC 20307-5100

c. NAME OF PRINCIPAL INVESTIGATOR

Sadoff, J

d. TELEPHONE NUMBER (include area code)

(202)-576-3759

21. GENERAL USE

FINA

MILITARY/CIVILIAN APPLICATION H

f. NAME OF ASSOCIATE INVESTIGATOR (if available)

Bancroft, W

g. NAME OF ASSOCIATE INVESTIGATOR (if available)

Kaufman, B M

22. KEYWORDS (Precede EACH with Security Classification Code) (U) lab animals; (U) mice; (U) RamV; (U) immunochemistry; (U) Soman; (U) monoclonal antibodies; (U) nerve agents

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23(U) Development of passive and active immunization for protection against nerve agents in humans. Nerve agents represent a serious threat to combat military personnel.

24(U) Nerve agents such as Soman and Soman analogues will be covalently coupled to protein carriers such as tetanus toxoid, and adjuvants such as gram negative bacterial membrane proteins, detoxified gram-negative lipopolysaccharides. These vaccines will be tested in mice for potency and used for vaccination of mice to produce monoclonal antibodies. Human monoclonal antibodies of high affinity will be produced by in-vitro vaccination of human lymphoid cells followed by fusion to long term human myeloma lines. Human monoclonal antibodies will be tested for potency. Tissue culture techniques for large scale production of human monoclonal antibody for human use will be explored. Recombinant DNA approaches to large scale production of antibody for human use will be explored.

25(U) 83 10-84 09 A new O-methoxy analogue of Soman was coupled through an amino group which had been inserted on the pinacolyl moiety to a succinylated protein. Screening of 130 clones yielded six hybrids producing monoclonal antibody highly reactive with the analogue. Affinity measurements against Soman are being determined at USAMRICD. In collaboration with contractors at Georgetown University using these conjugates a monoclonal with high affinity has been found. This antibody does not react with dipinacolyl Soman and therefore sees the reactive phosphinate group. A second analogue designed in collaboration with USAMRICD when coupled to protein under anhydrous conditions has the exact structure of the reactive portio of Soman and has been stable and non-toxic enough to immunize mice when mixed in Freund's adjuvant. Several clones have been produced using this new conjugate which are being tested for reactivity. (For technical report see Walter Reed Army Institute of Research Annual Report, 1 Oct 83 - 30 Sep 84).

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

Project 3M161102BS11 CHEMICAL AGENT EFFECTS AND ANTIDOTES

Work Unit 232 Immunochemistry of Nerve Agents

Investigators:

Principals: Jerald C. Sadoff, M.D., COL
Bennett Kaufman, Ph.D.
Robert Seid, Ph.D.

Problem:

Organophosphate nerve agents are a serious threat to armed forces and civilians throughout the world. The principle chemical warfare nerve agent is SOMAN. Its lethality is related to its high affinity for acetylcholinesterase combined with its ability to "age" i.e. lose its pinocolyl moiety after binding. This property makes it extremely difficult to displace from acetylcholinesterase with pharmocologic agents. Our approach is to produce a high affinity monoclonal antibody that could be used prophylactively to prevent SOMAN from reaching acetylcholinesterase. Prior to the institution of this project only low affinity monoclonals (affinity approximately $5X10^6$) which bound primarily to the pinacolyl end of SOMAN had been produced. New strategies for the development of high affinity monoclonals were undertaken.

Progress

Previous conjugates used for immunization of mice consisted of covalent coupling of chlorosoman through its phosphate moiety to spacer compounds that were then attached to carrier molecules. The active fluorine atom did not exist in these components. A methoxy group was substituted for the Fluorine and an amino group was placed on the pinacoly group. A succinamide spacer was used to couple this methoxy soman analogue to keyhole limpet hemocyanin through the amino group. Phosphorous 31 nuclear magnetic resonance (NMR) and elemental analysis confirmed a covalent coupling ratio of 20:1 hapten to carrier molecules. This compound was used to immunize mice and fusions were performed. Six monoclonals highly reactive with the conjugate but unreactive with carrier were identified at Walter Reed and their affinity for SOMAN is being tested. Eight monoclonals reactive with SOMAN were produced in collaboration with Dr. Tran Chen at Georgetown University who is an USAMRDC contractor. One of these has been shown to react directly with SOMAN with an affinity of $5X10^{-8}$ M which is two orders of magnitude greater than any previous antibody for SOMAN. Preliminary data from Dr. David Lenz USAMRICD indicates that this monoclonal is capable of neutralizing the ability of SOMAN to inactivate acetylcholestrase *in vitro*. This monoclonal does not react with dipinacolyl SOMAN indicating it is directed at the phosphate end of the SOMAN molecule.

A second conjugate designed in conjunction with Dr. Clarence Brimfield USAMRICD utilizes a difloro compound in its synthesis producing a conjugate that has the exact structure of SOMAN with the fluorine intact. This compound has been injected into mice in the presence of Freund's adjuvant. The mice have survived and produced antibody reactive with the methoxy SOMAN conjugate. Monoclonals have been produced but not tested as yet. The potential need for human as opposed to mouse monoclonals as prophylactic agents led us to develop a system for generation of human monoclonal antibodies. We have produced 16 stable human monoclonal antibodies, four of which are directed against the protective antigen of Anthrax. We therefore have the capability to produce human monoclonal antibody.

Future Plans

The high affinity anti-SOMAN mouse monoclonal generated thus far will be tested for prophylactic efficacy in animal models. More monoclonals using the two new conjugates will be generated. On a theoretical basis since 1 of 14 monoclonals had very high affinity we should be able to find monoclonals of even higher affinity if several hundred to several thousand are examined. Collaborative work with contractors will permit us to clone by recombinant means the high affinity monoclonal against SOMAN. Site specific mutagenes is to improve affinity and construction of a more variable human constant region hybrid for use in humans will be attempted.

There is good evidence from clinical trials utilizing monoclonal antibodies for imaging and therapy that mouse monoclonals when properly purified can be used safely in humans. Although antimouse antibody sometimes develops, anaphylactic and other serious reactions have not been seen. If the proper monoclonal can be found that offers a high degree of protection in animal models against SOMAN serious consideration should be given to its use in the field.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 300029	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS11	EB	233 WWGV
b. XXXXXXXXXX				
c. XXXXXXXXXX	STOG 82/83-6.20			

11. TITLE (Precede with Security Classification Code)
(U) Nerve agent antidote screening with invertebrate bioassay systems

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE 82 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	102
85	3.0	104

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, D C 20307-5100	b. ADDRESS Div of CD&I Washington, D C 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Andre, R G
d. TELEPHONE NUMBER (include area code) 202-576-3551	d. TELEPHONE NUMBER (include area code) 202-576-3719
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Wirtz, R A g. NAME OF ASSOCIATE INVESTIGATOR (if available) Golenda, C F

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Nerve agents; (U) Antidotes; (U) Musca domestica; (U) Atropine; (U) Pyradlioxamine chloride; (U) Sarin; (U) Soman; (U) Tabun; (U) VS (U) RAMV

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Develop and evaluate arthropod bioassay systems for screening of potential chemical nerve agent antidotes. Identify the most promising systems, develop testing methodology and conduct comparative evaluations with existing mammalian systems. The development of effective protective measures against chemical warfare agents is a high priority project for the Army and the search for antidotes to the known chemical warfare nerve agents is an important part of that effort. Clearly, realization of the objectives in this work unit may result in dedevelopment of several rapid, inexpensive arthropod bioassay screening systems for antidotes to CW nerve agents.

24. (U) Conduct a literature review to identify and prioritize the most promising insect systems. Establish new laboratory and insect rearing facilities to support this work. Identify laboratories equipped for CW nerve agent research and obtain permission to conduct research in their facilities. Establish selected insect colonies, develop to conduct research in their facilities. Establish selected insect colonies, develop and refine application procedures, testing methodology and statistical analysis criteria. Conduct tests and evaluate results so that the most useful and effective screening systems can be brought on-line.

25. (U) 83 10 - 84 09 Estimates of the LD50 and 95 values were determined from the probit regression lines for DFP, atropine sulfate and 2-PAM Cl. Five minute pretreatments with 2-PAM Cl or 2-PAM Cl plus atropine sulfate protected flies from a challenge dose of DFP that was approximately the LD95 dose. The highest pretreatment dose of 30ug PAM plus 20ug of atropine gave greater protection than did doses of lower concentrations. It was found that flies poisoned with a LD95 dose of DFP recovered following post-treatment with 2-PAM Cl and atropine, and survivorship was associated with post-treatment time. For technical report see Walter Reed Army Institute of Research Annual Report 1 Oct 83 to 30 Sep 84.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

Project 3M161102BS11 CHEMICAL AGENT EFFECTS AND ANTIDOTES

Work Unit 233 Nerve Agent Antidote Screening with Invertebrate Bioassay Systems

Investigators:
Principal: Richard G. Andre, LTC, MSC
Associate: Robert A. Wirtz, CPT, MSC; Claudia F. Golinda, Ph.D.; Yesu T. Das, Ph.D. (NRC Fellow)

Problems and Objectives

Development of defensive measures against chemical warfare nerve agents, classified as acetylcholinesterase (AChE) inhibitors (1,2) is a high priority Department of Defense medical research and development effort. AChE is the enzyme which breaks down acetylcholine, the primary neurotransmitter in the mammalian cholinergic nervous system. The blockage of AChE by nerve agents results in the continued presence of excess ACh. This causes repeated firing of target receptors on neurons, muscles and glands which leads to the disruption of neural communication and eventually death (3). Chemical warfare nerve agents with this suspected mode of action include sarin, soman, tabun and VX (1,2). These compounds are nonreversible inhibitors which rapidly and permanently bind to AChE. They also undergo unimolecular dealkylation, a process referred to as"ageing" (4,5). This ageing results in a nonfunctional enzyme-nerve agent complex which cannot be activated using traditional antidote therapy. The suspected mode of action of nerve agents is similar to that of the organophosphate insecticides, which are also nonreversible AChE inhibitors (3).

Therapeutic agents that are currently available are not completely satisfactory in saving life and in reducing physical and mental decrements. The search for new and more effective therapeutic and pretreatment drugs continues. Because of limitations in conducting efficacy studies in humans when one is dealing with organophosphorus nerve agents, testing is almost entirely carried out with in vitro studies or animal models.

As new antidotes or pretreatment drugs are proposed, it will be highly desirable to have a rapid inexpensive bioassay screening system available for drug development. Bioassay systems using intact insects have several distinct advantages over existing models. Most insect systems are rapid and relatively inexpensive with only small amounts of test material and nerve agent required. This can be especially important when candidate antidotes are custom synthesized and available only in small quantities. The use of small amounts of nerve agent, determined on a ug/kg live body weight, is an added safety factor. Most assays can be brought on line quickly, as no complicated equipment or training are usually required. Costly animal and handling facilities and personnel can be eliminated as less expensive mass rearing procedures have been developed for the arthropods currently under consideration for use. The ability to use large numbers of test insects and/or large sample sizes makes these tests particularly applicable to statistical analysis.

The question as to the applicability of tests conducted on insects to mammalian systems is a valid one due to differences in major organ systems and detoxification, activation and transport mechanisims. However, similar modes of action of nerve agents are suspected in both insects and vertebrates and the presence of similar receptors, enzymes and metabolic systems supports this premise. Once test data are available results can be compared to those of existing noninsect bioassay models and in vitro data to determine the feasibility of using insect systems for antidote screening.

Progress

Considerable progress has been made during the last 12 months of this project. Two investigators have been hired, a research physiologist and an invertebrate neurophysiologist. The house fly rearing procedures have been refined and approximately 1000 flies per week are being produced for development of screening methodology and enzyme studies.

The following are the major accomplishments in the development of the house fly bioassay system for screening of potential nerve agent antidotes. The initial studies of this system were designed to compare the house fly model with existing rodent and nonhuman primate tests. Estimates of the LD50s and LD95s were determined from the probit regression lines of flies injected with diisopropyl fluorophosphate (DFP), atropine sulfate (AS), and 2-pamchloride (2-PAM) with results calculated as ng or ug per mg live weight as well as on a per fly basis. Pretreatment with 2-PAM or 2-PAM plus AS protected flies from a LD95 challenge dose of DFP. The addition of AS to 2-PAM significantly increased survival over flies pretreated with 2-PAM only. Survivorship was approximately 88% in the flies pretreated with antidotes and only 5% in unprotected flies. Post-treatment therapy with 2-PAM plus AS of flies injected with DFP also decreased mortality, with survivorship correlating with time after treatment. Administering the antidotes 1 hour after exposure to DFP resulted in a 86% survival rate, with rates of 54% and 15% for treatment 3 and 6 hours after DFP exposure, respectively.

Behavioral studies using acetylcholine antagonists as well as studies on the characterization of house fly acetyl cholinesterase (AChE) also have been initiated. Methodology for detecting AChE activity has been established in the laboratory and activity has been demonstrated in house fly extracts. Purification of house fly AChE has begun for detailed characterization of the enzyme and its subunits, and for a direct comparison with AChE of other animals, including humans.

The data obtained to date on the house fly bioassay system demonstrate an excellent correlation with the currently used rodent systems. Specific areas where this agreement exists include challenge and therapy treatments of test materials, protection times afforded by the combined use of AP and 2-PAM, and the relationship between post-therapy treatment time and survivorship. While more comparative testing is required, all indications are that the house fly bioassay system will meet the need for a rapid, reliable, inexpensive initial screening system for nerve agent antidotes and will augment currently used rodent and nonhuman primate systems.

References

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2. Heilbronn, E. and R. Tolagen. 1965. Toxogonin in sarin, soman and tabun poisoning. Biochem. Pharmacol. 14: 73-77.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 300294	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS11	ED	WWH9 234
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 82/83-6.2/1			

11. TITLE (Precede with Security Classification Code)
(U) Molecular Biology of Medical Defense Against Chemical Agents

12. SUBJECT AREAS
0601 Biochemistry 0603 Biology 0616 Physiology

13. START DATE 81 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C.In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE — EXPIRATION
b. CONTRACT GRANT NUMBER
c. TYPE — d. AMOUNT
e. KIND OF AWARD — f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	3.0	583
85	3.0	536

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Biochemistry
b. ADDRESS (include zip code) Washington, D.C. 20307 - 5100	b. ADDRESS Washington, D.C. 20307 - 5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Chiang, P K
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-1361
21. GENERAL USE FINA	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Doctor, B P
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Olenick, J G

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Organophosphates; (U) Antidotes; (U) Mode of Action; (U) Receptors; (U) Enzymes; (U) RAMV

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objective of this work unit is to study the medical/chemical defense of military personnel against severely intoxicating chemical agents with a view to defining the molecular basis of inhibited cellular processes.

24. (U) Tissue culture and/or live animal methodology will be developed to study the mode of action of chemical agent poisoning. Pharmacokinetics and metabolic profiles of protective or therapeutic drugs also will be studied. Selective enzymatic assays for chemical agents, antidotes and cellular targets will be developed. The relationship and ontogeny of certain enzymes, receptors, and neurotransmitters will be studied. Recombinanat DNA and gene cloning procedures will be explored to further define ontogenetic relationships and topology of reactive sites.

25. (U) 83 10 - 84 09 The metabolism of the cholinolytic agent aprophen (AP) was studied in rats, and the antimuscarinic activity of some metabolites was compared with that of AP in N4TG1 neuroblastoma cells. Five min after i.v. administration of $[^{14}C]$AP, a substantial portion of the radioactivity was detected in the brain, adrenals, liver, and kidneys. Elimination of AP was rapid, mainly by metabolism to diphenylpropionic acid (DPPA) and diethylaminoethanol (DEAE). Antimuscarinic activity, measured by the inhibition of binding of the antagonist $[^{3}H]$QNB, was pronounced with AP ($I_{50} = 1$ μM) in contrast to minimal inhibition with DPPA ($I_{50} = 1$ mM), DEAE ($I_{50} = 0.2$ mM) and the putative metabolite DEAE-acetate ($I_{50} = 0.8$ mM). The enzymatic formation of DEAE-acetate could be demonstrated by using choline acetyltransferase. The I_{50} values suggest that the anticholinergic activity of AP after administration is mainly caused by AP itself. For technical report, see WRAIR Annual Report 1 Oct 83 - 30 Sep 84.

DD FORM 1498 83 MAR — EDITION OF MAR 68 IS OBSOLETE.

PROJECT: 3M161102BS11 CHEMICAL AGENT EFFECTS AND ANTIDOTES

WORK UNIT: 234 Molecular Biology of Medical Defense Against Chemical Agents

INVESTIGATORS:

Principal: Peter K. Chiang, Ph.D.
Associate: Richard K. Gordon, Ph.D.; B. P. Doctor, Ph.D.; Leo Kazyak; William M. Bone, Ph.D.; SP5 George Miura, Ph.D.; Jarle Aarbakke, M.D. Ph.D.
Assistant: SP4 Felipe N. Padilla; Michelle M. Richard

The objective of this work unit is to study the medical/chemical defense of military personnel against severely toxic chemical agents with a view to defining the molecular basis of inhibited cellular processes. Tissue models are developed for investigating the modes of action of organophosphates and their antidotes on the enzymes and receptors of attendant cholinergic systems. Other physiological and neurochemical parameters that are affected will also be assessed. The following investigations were conducted:

1. Antimuscarinic Activity and Biotransformation of Aprophen:

The metabolism of the cholinolytic agent aprophen (AP) was studied in rats, and the antimuscarinic activity of some metabolites was compared with that of AP in N4TG1 neuroblastoma cells. Five min after i.v. administraton of [^{14}C]AP, a substantial portion of the radioactivity was detected in the brain, adrenals, liver, and kidneys. Elimination of AP was rapid, mainly by metabolism to diphenylpropionic acid (DPPA) and diethylaminoethanol (DEAE). Antimuscarinic activity, measured by the inhibition of binding of the antagonist [^{3}H]QNB, was pronounced with AP (I_{50} = 0.8 mM). The enzymatic formation of DEAE-acetate could be demonstrated by using choline acetyltransferase. The I_{50} values suggest that the anticholinergic activity of AP after administration is mainly caused by AP itself.

2. Molecular requirements for the muscarinic receptors of N4TG1 neuroblastoma cells.

We recently reported that there are 2 x 10^5 muscarinic receptors/cell with a K_D about 13 nM in N4TH1 neuroblastoma cells as determined by Scatchard plot analysis of [^{3}H]QNB (quinuclidinyl benzilate) binding. binding (Biochem. Pharmacol. 1983, 32, 2979). Two groups of compounds were examined for their ability to inhibit QNB binding to the muscarinic receptors of N4TG1 neuroblastoma cells. The first group consists of analogs of S-isobutyladenosine (SIBA), which are novel muscarinic agents. SIBA and S-isobutyl-3-deazaadenosine (3-deazaSIBA) have been shown to have a variety of biological effects, chiefly attributed to the hypothesis that they act as S-adenosyl-homocysteine analogs and consequently as inhibitors of methylation. However, it has lately become obvious that 3-deazaSIBA has effects that are unrelated to methylation reactions (Trans. Am. Soc. Neurochem. 1981, 12, 82; Biochem. Pharmacol. 1982, 31, 2111). The most potent inhibitors of QNB binding are 1-deazaSIBA and 3-deazaSIBA (I_{50} values of 5.2×10^{-5} M and 3.9×10^{-5} M, respectively). The parent compound, SIBA, and the carbocylic derivative, S-isobutyl-3-deazaaristeromycin are less active. There is a requirement for the S-isobutyl side chain and N7 of the purine because 3-deazaadenosine, 5'-methylthioadenosine and S-isobutyltubercidin are inactive. Substitution of the thioether with N (5'-isobutylamino-adenosine) and modification of the ribose to an acyclic (9-[(2-isobutylthioethoxy) methyl]-adenine) resulted in loss of activity. It is conceivable that the ribose ring assumes a conformation similar to the furan ring of muscarine, and the S-isobutyl group acts like the hydrophobic benzilate portion of QNB or atropine. The next type of compounds tested were QNB analogs: The quinuclidine portion can be substituted with diethylamino ethyl ester, e.g. aprophen, benactyzine and adiphenine, and still retain equal potency to inhibit QNB binding. Furthermore, the quinuclidine can also be replaced by either an alkane, H, or pyrrolidine at the N without losing their ability to inhibit binding. Additions to the quinuclidine increase the bulk and decrease inhibition of binding. The benzilate (diphenyl) portion can be replaced by quinidines or tricyclic structures and still retain inhibitory activity. Similar to the benzilate in QNB or atropine, a hydrophobic structure is apparently required for activity.

3. S-Isobutyladenosine (SIBA) Analogs as a New Class of Antimuscarinic Agents:

Compounds structurally related to S-isobutyladenosine (SIBA) have been examined for their ability to inhibit the binding of QNB to the muscarinic receptors. SIBA and S-isobutyl-3-deazaadenosine (3-deaza-SIBA) have been shown to have a variety of biological effects, based on the hypothesis that they act as analogs of S-adenosylhomocysteine and perhaps as inhibitors of methylation (Biochem. Pharmacol. 31, (1982) 2111). Analogs containing the S-isobutyl chain and a deaza-adenosine moiety are the most potent inhibitors of QNB binding. The I_{50} for 3-deaza-SIBA is 5.2×10^{-5} M. Methylthioadenosine analogs have no effect. Substitution of the ribose by a carbocyclic decreases the inhibition, and modification of the ribose to an acyclic results in a loss of inhibition. These results reflect competition for the muscarinic receptors since (1) increasing incubation time fails to further decrease QNB binding, and (2) 3-deazaadenosine (a proximate inhibitor of methylation) by itself has no effect on QNB binding.

4. Inhibitors of CDP-choline synthesis, action potential calcium and stimulus-secretion coupling:

The effects of putative transmethylation inhibitors were tested on stimulus-secretion coupling and neuro-transmitter secretion at synapses between neuroblastoma x glioma hybrid cells and myotubes. 5'-Deoxy-5'isobutylthio-3-deazaadenosine and 5'-deoxy-5'isobutyladenosine inhibited CDP-choline synthesis catalyzed by cholinephosphate cytidylyltransferase, (CTP:cholinephosphate cytidylyltransferase, ED 2.7.7.15) and thereby decreased the rate of phosphatidylcholine synthesis from CDP-choline, but did not affect the transmethylation pathway for phosphatidylcholine synthesis. These compounds also inhibited $^{45}Ca^{2+}$ uptake by hybrid cells medicated by voltage-sensitive Ca^{2+} channels, acetylcholine secretion at synapses, and signal transduction through cell membranes mediated by myotube nicotinic acetylcholine receptors. In contrast, 3-deazaadenosine or adenosine inhibited the transmethylation pathway for phosphatidylcholine synthesis, but had no effect on Ca^{2+} action potentials, acetylcholine secretion, or signal transduction through cell membranes mediated by nicotinic acetylcholine receptors. These results show that the stimulus-secretion coupling and secretion reactions studied are not dependent on phospholipid methylation and suggest that the activity of action potential Ca^{2+} channels and the rate of neurotransmitter secretion are functionally coupled to the rate of phosphatidylcholine synthesis via the CDP-choline pathway.

5. New Techniques in Mass Spectrometry for Analytical Toxicology:

With the advent of secondary ion mass spectrometry (SIMS), new ionization techniques have been developed that already have had an impact on analytical applications in mass spectrometry. Fast atom bombardment (FAB) is one of these new ionization principles that promises to extend mass spectrometry into areas that were formerly inaccessible, such as the analyses of quaternary amines, peptides, oligosaccharides and, in general, large, thermally labile or involatile macromolecules. Our investigations have centered on quaternary amines and peptides by FAB. Examples pertinent to toxicology will be presented, as well as applications of conventional ionization utilizing desorption chemical ionization (DCI) and HPLC/mass spectrometry. Analyses of metastable transitions through linked scan (B/E and B^2/E) applications will be discussed, inasmuch as we have been able to utilize this technology to elucidate ambiguous spectra of compounds.

6. FAB/MS Analysis of HI-6 and Its Degradation Products:

Severe poisoning by organophosphorus compounds (e.g. insecticides and nerve agents) occurs from the inhibition of cholinesterase activity, resulting in an accumulation of acetylcholine in the effector organs. When the accumulation occurs near the ends of motor nerves to skeletal muscle, the resulting neuro-muscular block causes such effects as weakness and fasciculation. The accumulation of acetylcholine is thought to cause the persistent depolarization of the end plate region, and the block appears to last until partial restoration of cholinesterase activity in the muscle occurs. In severe cholinesterase intoxication, a paralysis of the CNS and of the peripheral respiratory muscules occur, and results in death.

One of the more widely used therapeutic antidotes for organophosphate poisoning, atropine, treats the muscarine-like effects caused by accumulation of acetylcholine at postganglionic cholinergic nerve endings, smooth, cardiac muscle, and secretory glands (symptoms of vomiting, abdominal cramps, sweating, etc.).

Therefore, studies of drugs that reactivate acetylcholinesterase have been undertaken with the assumption that once the enzyme has been reactivated with such an antidote, it will perform its normal function of metabolizing the accumulated acetylcholine and the syndrome of poisoning should abate.

The present study has focused on one of the most promising new drugs for treatment of cholinesterase poisoning, HI-6. Clearly there is a need to assay not only the activity of HI-6, but also its stability at various temperature and pH values.

The objective of the present study was to obtain a convenient, reliable assay for this class of bispyridinium oximes used as potent therapeutic compounds for organophosphate poisoning.

Reported here are the first mass spectral data on the bispyridinium oxime, HI-6. Due to the ionic nature of the compound, EIMS and CIMS are unsuccessful in obtaining molecular weight and other essential mass data. FABMS was used successfully, as shown in the spectra below, to obtain mass information for use as a stability probe in conjunction with HPLC, UV, and NMR analysis. Studies in this laboratory (both completed and in progress) reveal a marked effect of temperature and pH on the degradation of HI-6. Some of these products are shown below.

We conclude that 1) FABMS is an effective probe in the analysis of certain bispyridinium compounds, 2) the stability of HI-6 exhibits a temperature and pH dependence due to the oxime and carboxamide moieties; this was concluded upon investigation of several analogues of HI-6, 3) FABMS may be used in the analysis of certain polycharged organic compounds, with the apparent reduction in the gas phase giving molecular weight information.

7. EI as the Ionization Method of Choice for Mass Spectral Analysis of Siloxane Polymers:

Objective: To analyze Siloxane polymers by Mass Spectrometry and be able to classify them according to the side chains on the silicon atoms. Two ionization techniques are evaluated for their ability to accomplish this type of analysis, EI and PID (FAB). Polymers are often mixtures with an average molecular weight outside the mass range of most mass spectrometers. Therefore, fragmentation patterns are used to deduce what the various side chains are in addition to the chain terminus.

Siloxanes: General Properties and Applications: Common feature is the siloxy chain or backbone which may contain several hundred silicon atoms the side chains (R_{1-4}) usually consist of one or two different substituents such as the following: methyl; phenyl, including halogenated phenyl groups; trifluoropropyl; cyanoethyl; alkyl groups up to 3 carbon atoms (more than 3 carbon atoms in an R group do not usually change the properties of the polymers in a positive way; therefore, few siloxane polymers contain chains longer than propyl groups); branched chain polymers are fairly common are indicated by another siloxy chain in place of an R-group; polymerization is often terminated with a trimethylsilyl (Si(Ch$_3$)$_3$ group; phenyl groups may be substituted for 1 or more of the methyl groups. Another terminal group is the silanol, (-SiMe$_2$OH). Siloxanes have been found useful in such diverse areas as cosmetics and pharmaceuticals, vibration damping, heat transfer fluids, dielectric fluids, lubricants, and even gas chromatography.

Results: The Dimethyl-Polysiloxanes are the simplest of the compounds and can be differentiated from other siloxanes by noting certain ions and ion pairs in the EI Mass Spectra. Intense ions occur at masses 73,147,221 and every 74 amu thereafter up to abut mass 517. Also conspicuous are a series of ions beginning at mass 207 and occurring every 74 amu up to mass 503. Together these series of ions appear as doublets 14 amu apart. The Branched-chain polymers are indicated by an increase in the high mass to low mass ratio (high/low) (high= low + 14) for each doublet in the series. The compositions of the ions are easily rationalized (as repeating SiO(CH$_3$)$_2$ units) even though their origins may be in doubt.

The Phenyl-substituted Polysiloxanes vary according to the degree of phenyl-substitution and this is reflected in the EI Mass Spectra in the positive ion mode. Phenyl-substituted polysiloxanes are indicated by the presence of intense ions at masses 135 and 197 presumably due to SIO(CH$_e$)$_2$ and SiO$_2$CH$_3$ respectively. An ion at mass 147 and which is present in polysiloxanes with only methyl-substitution can be used in conjunction with peaks at masses 135 and 197 to reflect the degree of phenylsubstitution. The ratio of ion intensities for masses 147 and 197 to 135 vs. the moles of methyl-phenyl-substitution follow general trends (see plots) which can be used to estimate the degree of phenyl-substitution in other polysiloxanes. Only the Dimethyl-Diphenyl-Polysiloxane did not fall on the plots as expected and this may be due to the silanol chain stopped unit (all others were trimethyl-chain-stopped).

The Dimethyl-Methylcyanoethyl Polysiloxanes are difficult to characterize by their positive ion EI Mass Spectra. The spectra of two polymers of this type yielded significantly different ions which could not easily be assigned. Negative ion mass spectral data may be more indicative of the presence of cyano-groups in polysiloxanes and studies along these lines is called for in the future. To date PI-EI-MS has been unsuccessful in detecting cyanoethyl-substituents in polysiloxanes.

The 3,3,3-Trifluoropropyl-polymethyl Siloxane has not been characterized by the positive ion EI Mass Spectrum. This is due to a lack of appropriate standards more than anything else. Again negative ion mass spectral data might be more promising.

A Tetrachlorophenyltrisiloxy, Dimethyl-Polysiloxane (Trimethyl chain stopped) polymer was available and mass spectral data obtained. In addition this polymer with Iron Octoate added and one with Zinc Dialkylthiophosphate were available.

PUBLICATIONS

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Pankaskie, M. C., Itoh, T., Kachur, J. F., Gordon, R. K., and Chiang, P. K.: Analogs of 5'-isobutylthioadenosine (SIBA) as novel antimuscarinic agents. J. Med. Chem., in press.

ABSTRACTS

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Aarbakke, J., Gordon, R. K., Brown, N. D., Ayala-Medina, R., Miura, G. A., Doctor, B. P., and Chiang, P. K.: Antimuscarinic activity of aprophen and its metabolites. IUPHAR 9th International Congress of Pharmacology (1984).

Chiang, P. K., Chang, Y. F., Gordon, R. K., and Pankaskie, M. C.: Molecular requirements for the muscarinic receptors of N4TG1 neuroblastoma cells. Society for Neurosci. Abstracts 10, 168.20m (1984).

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Bone, W., Marasco, J. and Doctor, B. P.: EI as the ionization method of choice for mass spectral analysis of siloxane polymers. 32nd Annual Conference on Mass Spectrometry and Allied Topics, San Antonio, Texas. May 31, 1984.

Kazyak, L. and Bone, W.: New Techniques in Mass spectrometry for analytical toxicology, 21st International Meeting of the International Association of Forensic Toxicologists, Brighton. England, Sept. 13-17, 1984.

Kazyak, L.: What Progress in the Computerization of Toxicology After 18 Years? The International Association of Forensic Sciences 10th Triennial Conference, Oxford, England, Sept. 18-15, 1984.

PRESENTATIONS

(1) Chiang, P. K.: National Heart, Lung and Blood Institute, NIH (1 October 1983): "3-Deazaadenosine analogs as probes for cellular functions."

(2) Chiang, P. K.: Chairman, "Cholinergic Pharmacology", FASEB 1984 Meeting St. Louis (April 3, 1984).

PROJECT 3M463750D808

MEDICAL DEFENSE AGAINST MILITARILY IMPORTANT DISEASES

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OC 6481	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750A	3M463750D808	BA	001 WWMC
b. CONTRIBUTING			AA	
c. CONTRIBUTING	CARDS			

11. TITLE (Precede with Security Classification Code)
(U) Phase II Antimalarial Drug Trials

12. SUBJECT AREAS
0615 Clinical Pharmacology 0603 Biology

13. START DATE 78 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	3.0	278
c. TYPE	d. AMOUNT	85	3.0	302
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Division of Experimental Therapeutics Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR HEIFFER, M H
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (301)-427-5393
21. GENERAL USE FINA MILITARY CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) SCHUSTER, B G g. NAME OF ASSOCIATE INVESTIGATOR (if available) SHMUKLARSKY, M J

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Clinical Pharmacology; (U) Phase II Efficacy; (U) Antimalarial Drugs; (U) Volunteers; (U) ~~RAD I~~ RAD II

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The technical objective of this work unit is to evaluate the efficacy of new antimalarial drugs in non-immune human volunteers experimentally infected with malaria. Studies are performed in support of the Army Antimalarial Drug Development Program, and are an essential part of each official Investigational New Drug (IND) submission.

24. (U) Normal male volunteers are recruited from the civilian and military (MRVS) population of the greater metropolitan Washington, DC, area by public advertisement. Each individual receives a thorough medical evaluation and must give his valid, informed consent before being permitted to participate in the study. As a study subject, the volunteer is admitted to an in-patient research facility at Ft. Detrick, inoculated with malaria and treated with the drug or drugs specified in the protocol for each study. Each volunteer is then observed for a sufficient period of time to ensure that he is cured of malaria and is free from any adverse effect from his participation in the study.

25. (U) 83 10 - 84 09 Phase II efficacy studies of WR 180,409 were completed in 22 human volunteers with experimentally induced P. falciparum malaria (Smith isolate). The lowest effective total oral daily dose was 750 mg. Phase II studies of WR 194,965$\cdot H_3PO_4$ were completed. Six subjects were enrolled in the study with 2 recrudescences at the 2250 mg level. Phase II studies of this drug have been discontinued pending pharmacokinetic analysis of Phase I data. The responsibility for studies of WR 638 in treatment of cystinosis has been transferred to the National Institutes of Health. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project 3M463750D808 MEDICAL DEFENSE AGAINST MILITARILY IMPORTANT DISEASES

Work Unit 001 Phase II Antimalarial Drug Trials

Investigators:
Principal: Melvin H. Heiffer, Ph.D.
Associate: LTC C. Pamplin, MC, LTC B. Schuster, MC, LTC J. Berman, MC, MAJ M. Shmuklarsky, MC, L. Fleckenstein, Pharm.D.
Assistant: SSG P. Barr

1. Description.

Phase II clinical studies of antimalarial drugs involve the evaluation of the efficacy of candidate drugs in a limited number of patients subjected to a controlled clinical infection with malaria. These studies are an essential bridge between tolerance studies in healthy, non-infected volunteers and a wide-scale study of the curative potential of a new drug in malaria patients. A major aspect of Phase II studies is a determination of a curative dose. Pharmacokinetic evaluations of candidate drugs in man are also performed as they are an essential prerequisite to dosage selection.

2. Progress.

The efficacy of WR 180,409 has been studied in Phase II work with 22 human volunteers who had experimentally induced *P. falciparum* malaria (Smith isolate). It was determined that the lowest effective total oral daily dose was 750 mg.

An initial Phase II study of WR 194,965$\cdot H_3PO_4$ has been completed. The purpose of this study is to determine the lowest total dose which is 100% effective in treating this multi-drug resistant isolate. The six subjects enrolled in the study showed 2 recrudescences at the 2250 mg level using three oral doses of 750 mg at 0, 12, and 24 hours. These studies have been temporarily suspended pending final pharmacokinetic analysis of Phase I data. The method for this analysis has been revalidated and 124 plasma samples measured.

3. Future Work.

The results of the Phase II study of WR 180,409 will be evaluated to determine if Phase III studies are warranted. If so, these will be initiated. The pharmacokinetic data will be assembled and evaluated to determine the appropriate dosing regimen for continuing the WR 194,965 studies. New drugs will be considered for additional Phase II studies as Phase I work is completed and evaluated.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA OG 2527	84 10 01	DD-DR&E(AR)636

3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750 A	3M463750D808	AD	002 WW05
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS			

11. TITLE (Precede with Security Classification Code)
(U) Evaluation of New Antiparasitic Drugs and Vaccines in the Tropics

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
79 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE FISCAL YEARS	a. PROFESSIONAL WORK YEARS	b. FUNDS (In thousands)
84	7.0	1,192
85	7.0	984

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME US Army Medical Component, AFRIMS
b. ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS Bangkok, Thailand
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR SODETZ, F J
d. TELEPHONE NUMBER (include area code) 202-576-3551	d. TELEPHONE NUMBER (include area code) 66-2-281-7776
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	e. NAME OF ASSOCIATE INVESTIGATOR (if available) Boudreau, E F Rosenbert, R M Echever P E Webster, H K HOKE, C H WARD, C

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Malaria; (U) Mefloquine; (U) Halofantrine (U) Monkeys; (U) Volunteers; (U) RAM I (U) Lab animals;

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The technical objective of this task is to establish the efficacy of new drugs for both prophylaxis and treatment of tropical infectious diseases of military importance The effect of conventional and experimental antimalarials in treatment, prophylaxis and transmission of drug resistant falciparum malaria will be determined.

24. (U) Army investigational antimalarial drugs are compared with standard drugs and new combinations of standard drugs in the treatment and prophylaxis of drug resistant falciparum malaria in hospitalized human volunteers. Advanced development and field testing of new techniques supporting this task will be accomplished. Candidate antimalarial drugs will be evaluated using simian malaria, as a model for human malaria.

25. (U) 83 10-84 09 Two prophylaxis studies were completed comparing mefloquine, chloroquine-fansidar, and doxycycline in a trial in the Royal Thai Army, and the second comparing mefloquine, chloroquine, and chloroquine-fansidar in Thai gem miners. Therapeutic and prophylactic trials were completed in animal models looking at doxycycline in leptospirosis. The malaria drug screening program continued using monkeys obtained from the U.S. and produced at AFRIMS. Chemotherapeutic trials of halofantrine and quinine-tetracycline in RTN Marines and RTA troops respectively were continued. The in vitro drug sensitivity testing as complement of the drug trials was also continued. Biochemical targets of drug development looking at ADA and the effect of 2'deoxycoformycin in a primate model was also continued. A mefloquine pharmacokinetic study in malaria infected and noninfected Thais has begun. A treatment study comparing 180409 to mefloquine in P. falciparum infected patients has been started. For technical reports see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83 - 30 Sep 84

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EDITION OF MAR 68 IS OBSOLETE

PROJECT NUMBER: 3M43750D808 MEDICAL DEFENSE AGAINST MILITARILY IMPORTANT DISEASES

Work Unit Number 002 Evaluation of New Antiparasitic Drugs and Vaccines in the Tropics

Investigators: COL Frank J. Sodetz, MSC; LTC George S. Ward; MAJ Ellen F. Boudreau, MC; MAJ Horace K. Webster, MSC; MAJ Michael R. Elwell, VC; MAJ Richard G. Andre, MSC; MAJ George S. Childs, MSC; CPT Ronald M. Rosenberg, MSC, CPT Lorrin W. Pang, MC; Katchrinnee Pavanand, M.D.; Markpol Tingpalapong, VC

Quinine-Erythromycin and Chloroquine-Erythromycin Treatment of P. falciparum in Thailand

PROBLEM: In Thailand P. falciparum malaria strains have been resistant to chloroquine and sulfadoxine-pyrimethamine since the 1960's and 1980's respectively. Currently the recommended treatment is the combination of quinine-tetracycline. Quinine to rapidly alleviate symptoms and intially clear the patient's parasites and tetracycline to effect a radical cure.

There have been few studies testing erythromycin as a P. falciparum antimalarial. In 1976, Warhurst showed efficacy, even syngery, in chloroquine-erythromycin treated rats with P. berghei, infected with chloroquine and quinine resistant strains (1). Recently a Thai pediatric investigator at Mahidol University tested the quinine-erythromycin combination in a referred population of children with P. falciparum malaria and cured 80% (12/15) (2). Because of potentially fewer side effects and lower cost an effective chloroquine-erythromycin treatment regimen would be valuable in controlling malaria in Thailand.

PROGRESS: In 20 Royal Thai Marines and volunteer soldiers admitted to Ft. Taksin in Hospital in Chantaburi were administered large doses of erythromycin, 3 grams daily for 10 days, combined with standard doses of chloroquine (1500 mg over 3 days), the cure rate was 0% (4 RIII, 6 RII). In combination with standard doses of quinine, 650 mg q 8 hr x 3 days, the same erythromycin regimen cured only 2 out of a second 10 (8 RI) patients.

The mean initial parasite count was 4778 per mm^3 in the chloroquine-erythromycin group and 10,630 per mm^3 in the quinine-erythromycin group. None of the chloroquine-erythromycin group cleared their parasites. Mean parasite clearance time in the quinine erythromycin group was 102 hr, with a range of 86.5-112.5 hrs. Eighty percent of patients cleared their fevers on the chloroquine erythromycin regimen despite a progression of parasitemia. In addition, the mean fever clearance time (FCT) on chloroquine-erythromycin of 58.6 hr was not significantly different from the mean FCT in the quinine-erythromycin group of 66 hr.

Combined side efects of nausea, vomiting, diarrhea, abdominal pain or tinnitus were seen in 60-70% of patients on either drug combination.

In-vitro drug sensitivity testing performed in both drug groups demonstrated that the infecting strains were both chloroquine and quinine resistant. In Eastern Thailand the regimens of chloroquine-erythromycin and quinine-erythromycin appear to have no appreciable effect against P. falciparum malaria, animal data notwithstanding.

FUTURE OBJECTIVES:

1. To elucidate the mechanisms of action of the antimalarial - antibiotic combinations. (i.e. quinine and tetracycline)

2. To validate the data gathered in previous investigations on the efficacy of the combination amodiaquine - clindamycin.

REFERENCES:

1. Warhurst, D.C., Robinson, B.L., Peters, W.: The chemotherapy of rodent malaria XXIV, the blood schizonticidal action of erythromycin upon Plasmodium berghei. Annals of Tropical Medicine & Parasitology 70(3):253-258, 1976.

2. Chongsuphajaisiddhi, T., Sabchareon, A., Phamorsri, P.: In vivo and in vitro sensitivity of falciparum malaria to antimalarials in Thai childrean 1979-1982 Malaria Research, Thailand 1983 p 88 publication of Malaria Division of the Ministry of Public Health, Devavesm Palace Bangkok, Thailand.

The Pharmacokinetics and Plasma/Erythrocyte Distribution of Mefloquine in Patients with Naturally Acquired Falciparum Malaria

PROBLEM: Baseline mefloquine pharmacokinetic data describing drug half life, peak concentrations, expected range of concentrations over time, and percent of the drug bound to the red cell versus binding to serum proteins has been compiled using healthy volunteers in western countries (US & Switzerland) (1, 2). Quinine, a structurally related antimalarial, has higher established initial levels during the time the red cell is parasitized secondary to slowed hepatic metabolism of the drug, resulting in a greater amount of quinine in the circulation (3). In a study done by A. Hall, initial levels of quinine in the malaria infected patient were twice those seen in healthy individuals (4). The purpose of this study to compare blood levels of mefloquine in the infected to non-infected subjects. Establishing ranges of drug levels at various times post treatment is essential in assessing clinically recrudescent cases and in pin-pointing truly resistant parasite strains.

PROGRESS: From 11 June 1984 to 15 September 1984, at Trat Provincial Hospital 10 uninfected control patients were administered a single oral dose of mefloquine either 750 mg or 1500 mg and carefully followed over a 67 day course which included a series of 18 blood levels and frequent queries concerning symptoms of drug intolerance. The weight range in this group of healthy Thai volunteers, (hospital workers) was 49-76 kg with a median of 56.5 kg.

90% of the uninfected subjects reported some post dosing symptoms. 70% experienced diarrhea (1-4 watery stools in the first 48 hr post dosing) 50% experienced dizziness, 40% related nausea, 10% had abdominal pain and 50% had multiple symptoms.

In a second group of *P. falciparum* infected patients, one half of the group receiving 750 mg and the other half received 1500 mg in a double blind randomized fashion, three out of a total of twenty planned patients were treated during this time interval.

All were cured. All had gastrointestinal side effects (1/3 nausea, 1/3 vomitng, 1/3 diarrhea). Initial parasite counts ranged from 17,472-71,370 per mm^3. Parasite clearance times ranged from 65-118 hr. Fever clearance times ranged from 56-64 hr. The patients were all in their twenties and ranged from 45-60 kg in weight. All were tapioca farmers who had been ill for 3-4 days prior to admission. All had a prior history of 3-4 malaria infections.

FUTURE OBJECTIVES:

1. To complete the study, requires treating 17 more *P. falciparum* infected patients with mefloquine, completing each patient's 67 day clinical follow-up, performing high performance liquid chromatographic analysis of plasma, RBC and urine specimens and compiling the pharmacokinetic data on each patient.

2. To analyze in vitro drug sensitivity profiles, on admission to the study, in each malaria infected patient.

REFERENCES

1. Desjardins, R.E., Pamplin, C.L., VonBredow, J., Barry, K.G., and Canfield, C.J.: Kinetics of a new antimalarial, mefloquine. Clinical Pharmacology & Therapeutics 26:372-379, 1979.

2. Schwartz, D.E., Eckul, G., Hartmann, D., Weber, B., Richard-Lenoble, D., Ekue J.M.K. and Gentilini, M.: Single dose kinetics of mefloquine in man. Chemotherapy 28:70-84.

3. Trenholme, G.M., William, R.L., Rieckmann, K.H., Frischer, H. and Carson, P.E.: Quinine disposition during malaria and during induced fever. Clinical Pharmacology & Therapeutics 19(4): 459-67, 1976.

4. Hall, A.P., Hanchalay, S., Doberstyn, E.B., and Bumnetpand, S.: Quinine dosage and serum levels in falciparum malaria. Annual Report SEATO Medical Research Laboratory p. 241-150, 1975.

The Treatment of P. falciparum Malaria with Halofantrine, A Phenanthrenemethanol

PROBLEM: In Eastern Thailand, multi-drug resistant *P. falciparum* malaria is a prominent concern. Less than 10% of patients with falciparum malaria on the Thai-Kampuchean border are still cured by Fansidar treatment (1). Mefloquine treatment is still 88-97% effective (2, 3), but with the commercial release of the MSP (mefloquine-Fansidar) combination this year in Thailand, resistance may soon be a problem.

Secondline drugs for mefloquine resistant patients must be developed. Halofantrine, a phenanthrenemethanol, is in a separate chemical class from mefloquine and quinine which are quinolinemethanols, therefore the development of cross-resistance might be delayed.

PROGRESS: Halofantrine HCL (WR 171, 669) was administered orally to 82 semi-immune subjects infected with *P. falciparum* malaria on the Thai-Kampuchean border in a randomized double-blind treatment trial comparing the efficacy of halofantrine to mefloquine.

Halofantrine HCL was curative in a split-dose single day oral treatment regimen in 89% (54/61) of patients receiving 500 mg every 6 hrs x 3 doses (3 dose regimen) and in 65% (13/20) of patients receiving 1000 mg followed 6 hr later by 500 mg (2 dose regimen). Mefloquine was curative in 60/64 patients (94%) when given as a single dose of 1500 or 1000 mg.

The 1000 mg mefloquine dose was 88% curative (22/25) while the higher 1500 mg dose was 97% curative (38/39). This was not a significant difference, due to small patient numbers.

There was no significant difference in cure rates between the halofatrine triple dose regimen and either mefloquine treatment (Fisher's exact test).

The mean parasite clearance time for the halofantrine 3 dose regimen was 76 ± 19 hr in 61 patients, 78.8 + 28.8 hr for 39 patients on a 1500 mg dose of mefloquine and 75.4 + 25.2 hr for 24 patients receiving a 1000 mg single dose of mefloquine.

The mean fever clearance times for all 4 groups was between 50-60 hr. No significant difference was found in fever clearance times or parasite clearance times for the 4 drug groups (student T test).

Post dosing side effects in the halofantrine group were not significantly different from those in either the high or low dose mefloquine groups. Though there was a trend toward less diarrhea and more asymptomatic patients in the halofantrine drug groups.

FUTURE OBJECTIVES:

1. To perform efficacy and pharmacokinetic studies of the new tablet formulation of the halofantrine HCl salt.

2. To determine the efficacy of weekly prophylaxis with halofantrine in comparison to mefloquine.

REFERENCES:

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2. Boudreau, E.F., Pang, L.W., Dixon, K.E., Webster, H.K., Thosingha, L., Phintuyothin, P. & Canfield, C.J. Comparable efficacy of halofantrine and mefloquine in the treatment of *P. falciparum* malaria on the Thai-Kampuchean border-Abstracts of The American Society of Tropical Medicine and Hygiene Meeting December 4-8, 1983.

3. Boudreau, E.F., Pang, L.W., Dixon, K.E., Webster, H.K., Pavanand, K., Canfield, C.J., Ua-umnuay, T., Supanantalerk, C., Chiperm, S., Keo-Ke-Jee, S. and Triparara, P. Comparable efficacy of mefloquine and the combination of quinine and tetracycline therapy for falciparum malaria in Eastern Thailand Abstracts of The American Society of Tropical Medicine & Hygiene Meeting December 2-6, 1984.

The Comparison of Mefloquine and Doxycycline as Clinical Prophylactic Agents for Falciparum and Vivax Malaria in Royal Thai Army Troops Assigned to the Thai-Kampuchean Border

PROBLEM: Effective antimalarial prophylaxis in many areas of the world is an area of uncertainty. Current controlled prophylactic trials are rare and decisions are often made using out-of-date information as guidelines in recommending prophylactic regimens. This study was undertaken to compare one widely used regimen chloroquine-Fansidar, to mefloquine and to a third, newly proposed prophylactic agent, doxycycline.

Doxycycline is used by the US military for diarrheal prophylaxis (1), leptospirosis prophylaxis (2) and for scrub typhus prophylaxis (3), thus with its broad usage in tropical areas, antimalarial efficacy would be extremely useful.

PROGRESS: This study was conducted from July, 1983 - October, 1983 during a 14 week period involving 321 Royal Thai Army and volunteer soldiers stationed along the Thai-Kampuchean border near Aranyaprateet. Twice weekly the principal investigtors witnessed dosing in the study patients with randomized double blinded medications and weekly supervised thick smear examination for malarial parasites. At the time of dosing, symptom histories were also obtained. Every attempt was made to locate each study subject on a semi-weekly basis. Absences were accounted for by vacations, patrols and assignments to bring supplies to the troops from town. Comparability of groups was confirmed and there was no significant difference between drug groups in absenses from the site, in the total number of half weeks each patient was dosed, age or weight. Semi-weekly side effects were also not significantly different between the 3 drug groups. Hematology and biochemical parameters were examined every 6 weeks and were not significantly different before & after medication for all groups.

In this malaria prophylactic study, life table analysis was used to determine the relative protection rates of the drug groups at the end of the 12 wk exposure period. Background malaria incidence using untreated patients could not be determined, in this study. Mefloquine (250 mg weekly) was shown to be a superior *P. falciparum* and *P. vivax* prophylactic to both a doxycycline (200 mg twice weekly) and a chloroquine (300 mg weekly) plus Fansidar (25 mg P + 500 mg S weekly) regimen. In the entire study population of 321 soliders there were no falciparum or vivax infections in the mefloquine group (n = 109) compared to 14 falciparum and 8 vivax infections in the doxycycline group (n = 108) and 23 and 1, respectively, in the chloroquine + Fansidar group (n = 104). This included infections which may have occurred during unsupervised medication administration. Using data generated only during periods of supervised medication administration, mefloquine was again shown to be a better suppressive prophylactic than the other 2 regimens against *P. falciparum* ($p < .026$) and against *P. vivax* ($p < .045$). Excluding any parasitemias which occurred during the first 10 days of drug administration (one prepatent period), thus including any exo-erythrocytic action of the drugs, melfoquine was still shown to be superior in the prevention of falciparum malaria ($p < .01$) though the vivax infection rate for this set of data was too low to show a significant difference among groups.

This is a preliminary report. Definitive data analysis is pending complete re-reading of all the malaria smears.

FUTURE OBJECTIVES:

1. To elevate a daily dose of 100 mg doxycycline for antimalarial prophylactic efficacy.

2. To test other commonly used prophylactic agents such as proquanil or Maloprim for their protective activity against P. falciparum and P. vivax in Thailand.

3. To develop better methods of determining the exposure risk of a population prior to initiation of a prophylactic study.

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3. Olson, JG, Bourgeois AL, Fang RC, Coolbaugh JC, and Dennis DT (1980) Prevention of scrub typhus: Prophylactic administration of doxycycline in a randomized double blind trial. American Journal of Tropical Medicine and Hygiene 29(5) pp 989-997.

The Treatment of P. falciparum Malaria with a Combination of Quinine and Tetracycline

PROBLEM: The P. falciparum drug sensitivity profiles from Thailand constitute some of the world's most resistant strains. The Thai Ministry of Health through its Malaria Division tested the efficacy of quinine 600 mg tid x 3 days and tetracycline 500 mg bid x 7 days and found a 97% efficacy among 30 patients in Surin Privince, Northeastern Thailand in 1982. This compared favorably with quinine 600 mg tid x 6 days and tetracycline 500 mg bid x 7 days which had a 100% cure rate among a second group of 30 patients from the same area (1). From 1980-81, a 90 patient study using Q_3T_7 in 5 diverse regions of Thailand (the Burmese border, the Cambodian border, the North, the South and the Northeast) resulted in a 95% cure rate (2). However Professor Tranakchit, School of Tropical Medicine, Mahidol University reported only 59% efficacy on the same 3 days of quinine and 7 days of tetracycline regimen from 1981-1982 among 21 patients from Prachinburi Province, Eastern Thailand in a study of hospitalized patients with 35 day inpatient followup (3). This conficting data prompted our investigation.

PROGRESS: This randomized antimalarial treatment efficacy study was conducted in the Thai marines, Army and paramilitary stationed along the Cambodian border from November, 1982 - July, 1984. Mefloquine as a 1500 mg single dose (MEF) was compared to both quinine 650 mg q 8 hr x 3 days plus tetracycline 500 mg q 8 hr x 7 days (high dose Q_3T_7) and to quinine 650 mg loading dose followed by quinine 325 mg q 6 hr x 3 days plus tetracyline 250 mg q 6 hr x 7 days (low dose Q_3T_7). All patients were hospitalized for 21 days in a non-malarious area and followed for 28 days. Initial parasitemias ranged from 1400 per mm^3-128,000 per mm^3. All patients had malaria symptoms on admission.

	FCT (hrs)	PCT (hrs)	Cure Rate	Type of recrudescence	IPC (per mm^3)
Mef (n=62)	60.7	77.0	89%	5 RI, 2 RII	31,768
High Dose Q_3T_7 (n=55)	69.0	95.7	93%	4 RI	30,468
Low dose Q_3T_7 (n=52)	69.0	102.6	94%	2 RI, 1 RII	26,332

Both patients with RII clinic responses to mefloquine had sensitive parasites (ID$_{50}$ 1.35 & 6.04 ng/ml). Poor absorption was documented by blood levels. The parasite clearance time for mefloquine was significantly less than for either Q-T group ($p < .01$ Student T Test). Cure rates were not significantly different by Fisher's exact test. No significance was found in the incidence of diarrhea, nausea or vomiting between groups. There was no decrease in side effects in the low dose Q_3T_7 group but efficacy remained the same. If compliance can be assured, Q_3T_7 continues to be a highly effective treatment for *P. falciparum* in Thailand.

FUTURE OBJECTIVES:

1. To determine the efficacy of a quinine-doxycycline treatment regimen.

2. To develop and utilize a tetracycline in-vitro drug sensitivity testing in the field, in addition to obtaining peak and trough tetracycline levels.

AVAILABLE

REFERENCES:

1. Pinichpongse, S. Field studies in Thailand of melfoquine, mefloquine/Fansidar, and mefloquine/primaquine 1981-1983. Abstracts of the Southeast Asian Regional Collaborative Studies on Drug Resistant Malaria - Jakarta, Indonesia 2-6 May, 1983.

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The Treatment of *P. falciparum* Malaria with WR 180,409, a Pyridinemethanol

PROBLEM: The rapid spread of multidrug resistance to *P. falciparum* malaria in Southeast Asia creates a continuing need for the development of newer antimalarial drugs. In the 1970's mefloquine proved to be 96-100% curative against all resistant strains, when administered as a single dose of 20 mg/kg (1, 2). Currently that same mefloquine drug regimen cures only 85-90% of hospitalized *P. falciparum* patients (3, 4). WR 180,409, known as ENPIROLINE, now enters phase III clinical testing as an alternative single day treatment regimen for resistant *P. falciparum* malaria.

Phase II testing of this drug at USAMRIID in 22 healthy non-immune volunteers blood innoculated with Vietnam Smith strain *P. falciparum* cured 21/22 patients (5). The only failure was on a 500 mg single dose. The lowest dose to result in a 100% cure was 500 mg followed twelve hours later by 250 mg. Nausea, vomiting and diarrhea were observed post drug treatment, but all cases were transicent and mild. The objective of this field study is to assess the tolerance and efficacy of ENPIROLINE as a split dose over a single day against naturally acquired *P. falciparum* malaria in an area endemic for multi-drug resistant malaria along the Thai-Kampuchean border.

PROGRESS: From 11 June - 20 July 1984 the first 11 patients were admitted at Ft. Taksin, Chantaburi in a double blinded randomized treatment trial comparing, ENPIROLINE, 2 dose single day therapy, to mefloquine single dose therapy. These subjects were Royal Thai marines and volunteer soldiers with naturally acquired *P. falciparum* malaria who presented with: Parasite counts between 1000-100,000/mm^3, no mixed infections and, no complications such as prolonged vomiting, anemia (Hct < 25), CNS, pulmonary, renal or cardiovascular compromise. These patients were 20-50 yr of age and 40-70 kg in weight and received no prior antimalarials prior to admission. They were observed by AFRIMS personnel as the medication was administered and kept under careful clinic observation during hospitalization in a non-malarious area for a 28 day period post treatment.

Two recrudescences occurred in the first 11 patients (1 at day 21 and the second at day 10). Only in the recrudescent patients was the treatment code broken by an unbiased observer. Both mefloquine and ENPIROLINE accounted for 1 recrudescence each. One patient was removed from the study due to concurrent TB therapy. Drug levels post treatment in both patients have not yet been analyzed.

The intial parasite count for the 10 patients, which included both mefloquine and WR 180,409 treatments, ranged from 9700-84,660 per mm^3, parasite clearance time ranged from 49 hr-119 hr with a median of 72 hr and fever clearance times ranged from 27-75 hr with a median of 45 hr. Side effects occurred in 6/10 study patients. These consisted of nausea, vomiting or diarrhea within the first 48 hours.

The study was halted after 5 weeks due to temporary diasapproval by the HSRRB for an incomplete patient information form. Continuation of the study should begin in October, 1984.

FUTURE OBJECTIVES:

1. To determine, by in vitro drug sensitivity testing, the degree of antimalarial drug cross-resistance which occurrs between mefloquine and WR 180,409.

2. To assess what percentage of clinical failures are due to inadequate drug levels.

3. To determine if the GI side effects with ENPIROLINE are less than those with mefloquine.

4. To determine field efficacy as culmination of the development of this antimalarial drug.

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The Comparison of Chloroquine, Chloroquine-Fansidar, and Mefloquine as Clinical Prophylactic Agents for P. falciparum and P. vivax Malaria in Thai Gem Miners Along the Thai-Kampuchean Border.

PROBLEM: In Thailand, P. falciparum malaria has been resistant to chloroquine since the 1960's and to Fansidar since the 1980's.

While studies from the 1970's show a Fansidar treatment success rate between 80-90% (1,2,3) a recent study by Dixon (1979-1980) which included Thai marines assigned to the Thai-Kampuchean border show a treatment success rate of only 9.1% (2 tablets) and 19.4% (3 tablets) (4). This rapid increase in the failure rate may have been due to drug resistance development with widespread use of Fansidar but more likely, as pointed out by Dixon, his study included many cases of "Kampuchean" strain *P. falciparum* malaria acquired near the border. That Kampuchean *P. falciparum* represents a different strain, is supported by Johnson's study in 1980 where Fansidar treatment failed in 9 of 9 Khmer patients in a Thai refugee camp (5). Though the origin of this strain is uncertain, Johnson felt that it probably was imported from Kampuchea.

Thai nationals frequently cross the Kampuchean border near the town of Borai, Thailand for the purpose of gem mining. Small groups of several to a dozen men travel a maximum of 40 km into Kampuchea to work in this hilly region for a 7-21 day period before returning to Thailand for supplies. These miners have a very high monthly incidence of malaria. With both resistant strains of *P. falciparum* and high transmission rates for malaria, this area and population group are ideal for a definitive prophylactic study.

PROGRESS: July, 1983 - March, 1984 a randomized double blind prophylactic trial in Thai gem miners was conducted to determine the protection rate of 3 drug regimens against *P. falciparum* and *P. vivax* malaria along the Thai-Kampuchean border. Gem miners were chosen as the study population due to the high incidence of malaria in this group. Selection criteria included: age > 21 yrs, a negative history of prophylaxis, a past history of mining for > 3 mo, intention to remain in the area for 6 months and a negative malaria smear. Duration of individual participation was 14 weeks. Results: Of the 502 participants in this study, who were seen every 2 weeks and dosed orally, 223 received mefloquine 500 mg, 165 received chloroquine 300 mg base plus Fansidar (1000 mg sulfadoxine and 50 mg pyrimethamine) and 114 received chloroquine 300 mg base. In the intervening weeks, the patients in the chloroquine-Fansidar and chloroquine groups self administered chloroquine 300 mg base and the mefloquine patients self administered identical placebo tablets. Life table analysis showed protection rates of: 92% for the mefloquine group, 13% for the chloroquine-Fansidar group and 9% for the chloroquine group. The Breslow test comparing the 3 survival curves (measuring protection from malaria) had a p value < .0001. The Breslow test comparing protection in chloroquine-Fansidar to that in the chloroquine group showed no significant difference (p = .39). These results show that standard regimens prescribed for prophylaxis are not longer effective for multidrug resistant strains of Plasmodium falciparum along the Thai border, although mefloquine was demonstrated to be highly effective.

Since the original analysis of the results of this study. The principal investigators have re-examined every malaria smear in each prophylactic drug group. An additional 29/223 patients smears in the mefloquine group were rare positives during prophylaxis and remained clinically undetected while the slide continued have rare parasites (less than 1 falciparum parasite per 100 WBC on the thick smears) or converted to negative during the study. In contrast only 2 patients with rare positive smears per group were missed in either the chloroquine or chloroquine-Fansidar group. This indicates a 8.8 times greater chance of difficult-to-detect rare parasitemias occuring during mefloquine chemoprophylaxis. There was no greater incidence of symptoms of malaria in the rare (+) patients than in the completely (-) patients. This finding of rare parasitemias may be interpreted in one of three ways:

1. Rapidly increasing resistance to mefloquine since the earlier prophylactic study by Pearlman and Doberstyn in 1977 where only 3/487 subjects who received mefloquine at either 180 mg per week, 360 mg per week or 360 mg every 2 wks became parasitemia during a 6 month period (6). Recompiling the relative malaria protection rates from a newly constructed life table which eliminates all the rare parasitemias as malaria positives results in: a markedly reduced mefloquine protection rate of 60% with 8% for the chloroquine-Fansidar group and only 3% for the chloroquine group.

2. Poor absorption of the biweekly 500 mg dose of mefloquine.

3. Rare parasitemia is a commonly observed phenomenon following mefloquine treatment or prophylaxis. It has been reported in Thailand and Burma during large scale treatment trials, rare, asymptomatic parasitemia appears and disappear without any intervention (7).

FUTURE OBJECTIVES:

1. To perform a pharmacokinetic study of 250 mg mefloquine per week compared to 500 mg mefloquine biweekly over a 16 wk course, in healthy Thais.

2. To add sequential mefloquine blood level monitoring in randomly selected patients during a future mefloquine prophylactic efficacy study.

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Comparative Strain Susceptibility of Anopheles to Plasmodial Parasites

PROBLEMS: The objectives of this investigation are as follows: a. to determine and compare the susceptibility of primary and potential secondary vectors of malaria to Plasmodium parasites; b.

to delineate the development of malaria parasites in Anopheles species with varying degrees of susceptibility; and c. to observe the feeding behavior of colonized vectors of human malaria under laboratory conditions.

PROGRESS: Studies on the susceptibility of various anophelines to human malaria continued this year at the Malaria Control Center, Tha Muang, Kanchanaburi Province. Anopheles dirus A was compared to other colonized Thai Anopheles taxa in paired-feeding experiments. Thirty-two patients infected with falciparum gametocytes successfully infected dirus A. Over three-quarters of the total mosquitoes dissected had developed oocysts, but only forty percent had positive salivary glands. Half of the other mosquitoes (dirus B and C, maculatus A and B) became infected and, of those, forty-one percent had sporozoites. Results from forty-five paired feeds on Plasmodium vivax patients showed that the mosquitoes were more susceptible to this parasite. Overall, dirus A was more susceptible to human malaria parasites than the other taxa tested.

FUTURE OBJECTIVES: This study will be continued next year in order to test the susceptibility of the new species in the balabacensis complex and the maculatus complex. Known genetic lines will be used to determine the importance of these sibling species in the natural history of malaria in Thailand.

Antimalarial Drug Resistance

PROBLEM: Mefloquine hydrochloride, a quinoline methanol, is a new single-dose blood schizontocide developed by the US Army Antimalarial Drug Program. Currently mefloquine is the only non-combination therapy for treatment of multi-drug resistant P. falciparum. Only recently has mefloquine been introduced to the field, and only in Thailand. Resistance to mefloquine would not be expected given the drugs limited use in the field. Nonetheless during the past two years we have confirmed several RI and RII type treatment failures with mefloquine in Thailand. The importance of mefloquine to the treatment of multi-drug resistant P. falciparum malaria makes imperative the need to study the emergence of natural parasite resistance to mefloquine. It is also essential that we try to understand the genetic and biochemical mechanisms of mefloquine resistance.

PROGRESS: As a first step towards obtaining a genetically defined P. falciparum parasite we have cloned and characterized the pre- and post-treatment parasite isolates from a Thai patient with a confirmed RI type treatment failure to mefloquine. *In vitro* susceptibility testing showed the pre-treatment isolate (CH150D0) to have a mefloquine ID50 of 2.13 ng/ml (susceptible) when cultured directly from the patient. Enzyme typing of CH150D0 for glucose phosphate isomerase (GPI) revealed a mixture of electrophoretic forms with GPI type I in greater proportion than GPI type II. The primary isolate gave a mixed response on testing against a panel of 18 monoclonal antibodies. CH150D0 appeared thus to be a heterogenous population of parasites in which mefloquine sensitive forms were the predominant drug phenotype. The isolate collected at recrudescence on day 21 post-mefloquine treatment (CH150D21) had a mefloquine ID50 of 15.24 ng/ml. Only one enzyme form, GPI type I, was observed for CH150D21. A uniform response to monoclonal antibodies was demonstrated. These observations on CH150 D21 indicated a homogenous parasite population characteristically resistant to mefloquine. Clones from the pre-treatment isolate were predominantly susceptible to mefloquine *in vitro* and were distinguished by a separate pattern of GPI enzyme types and appeared heterogenous in their reaction with monoclonal antibodies. The post-mefloquine treatment clones were uniformly resistant to mefloquine *in vitro* and homogenous for both enzyme type and reactivity to strain specific monoclonal antibodies. It appears, therefore, that there is considerable genetic diversity in natural *P. falciparum* isolates in Thailand. An additional observation in this study was that all the clones from CH150D21 were uniformly cross-resistant to halofantrine *in vitro.* Halofantrine is currently undergoing Phase III clinical testing in Thailand.

RECOMMENDATIONS: This study suggests that the potential for mefloquine resistance already exists in the indigenous *P. falciparum* gene pool. Once exposure to mefloquine is encountered the resistant forms grow with selective advantage and produce a stable drug phenotype. The implication for antimalarial chemotherapy from this study for mefloquine (and structurally related antimalarials, e.g., halofantrine) is ominous. There is an urgent need to further characterize and use the clones obtained in studies such as this one to investigate the biochemical and genetic basis of drug resistance in *P. falciparum.* This project should be continued with the highest priority.

Field Evalaution of New Antimalarial Drugs

PROBLEM: The emergence of strains of *Plasmodium falciparum* which are resistant to treatment with chloroquine, pyrimethamine-sulfadoxine (Fansidar), and quinine has emphasized the need to moniter the drug sensitivity patterns of field isolates. *In vitro* evidence of low drug susceptibility or patterns of cross-resistance between standard and new compounds may influence the selection of potential new antimalarials for further development. Evidence from murine models has suggested that patterns of resistance to several of the new antimalarials (mefloquine, halofantrine, WR 180,409) may be related to cross-resistance with quinine related to a common aminoalcohol structure. A number of methods have been reported to support field studies of drug susceptibility. However, the introduction of new antimalarials to standard test systems may require substantial effort to identify and overcome potential limitations such as adsorption of the drug to plastic culture plates.

PROGRESS: Drug susceptibility patterns of a series of isolates of *P. falciparum* from the region of the Thai-Burmese border were determined using a modified *in vitro* field assay system. Standard and new compounds were prepared as solutions and serially diluted in microtiter plates in the field. Depending on observed levels of susceptibility initial concentrations were adjusted as appropriate. The time of incubation was also varied to permit optimal maturation of ring stages to schizonts. Thick films were prepared and schizonts/200 parasites were scored. This modified system is highly portable and is sensitive to low parasitemias. The results of this study indicate a high level of variation of susceptibility among the isolates to all the compounds. This population of parasites had high average ID_{50}s (ng/ml) for chloroquine (127), quinine (206), and pyrimethamine (2300) and appeared to be susceptible to new compounds which are in various stages of development: mefloquine (5.93), halofantrine (3.24), WR 180,409 (3.78), and WR 194,965 (7.58). Patterns of rank-order correlations in this study did not support the hypothesis that cross-resistance is related to the aminoalcohol group.

RECOMMENDATION: Additional parallel studies should be conducted in different areas of Thailand and subsequently in areas of Southeast Asia to determine the geographical extent of drug resistance to the standard antimalarial compounds. This should also provide data on the consistency of patterns of cross-resistance with new compounds. In addition other promising compounds may be evaluated in the field environment. Studies on the structure activity relationship of drug resistance may be evaluated by the study of compounds with appropriate structural modifications.

In Vitro Assay of Pyrimethamine-Sulfadoxine Activity

PROBLEM: The combination of pyrimethamine and sulfadoxine (Fansidar) has been widely used as a therapeutic and prophylactic agent for chloroquine and pyrimethamine-resistant strains of *Plasmodium falciparum.* However, the efficacy of this drug has been diminishing in regions of southeast Thailand as well as in South America and Africa. The lack of a suitable *in vitro* assay system has precluded onsite monitoring of the changing sensitivity of the isolates and evaluation of the geographic extent of the resistance to Fansidar. There is also little evidence to suggest the role of resistance of each component to the resistance of the combination in patients. A major limitation of the standard system to assaying Fansidar activity has been the high levels in the standard medium of p-aminobenzoic acid (PABA) and folic acid which compete with the antimalarial activity of the sulfadoxine and pyrimethamine.

PROGRESS: The *in vitro* susceptibility of a series of field isolates of *P. falciparum* from the region of the Thai-Kampuchean border to pyrimethamine, sulfadoxine, and their combination was determined using a microtiter test system and medium deficient of PABA and folic acid. Two-fold serial dilutions of pyrimethamine ranging from concentrations of 8.0 to 0.125 uM and sulfadoxine ranging from 800 to 50 uM were evaluated for antimalarial activity. Viability was based on the maturation of ring stages to normally-appearing schizonts. Tested singly the parasites were resistant to pyrimethamine having an average ID_{90} of 3.82 uM and were highly resistant to sulfadoxine with all isolates having an ID_{90} greater than 800 uM. Analysis of the data showed potentiation of the antimalarial activity of the pyrimethamine approximately 4.4 times at a concentration of 0.8 uM of pyrimethamine and 80 uM of sulfadoxine. The concentrations in the *in vitro* assay system which were equivalent to the maximum concentrations in human plasma of sulfadoxine and pyrimethamine (375 uM and 1.44 uM, respectively) following a two tablet regimen were equivalent only to an ID_{98}. This may, in part, account for the diminishing efficacy of the drug combination for the treatment of infections of *P. falciparum* in the region of the Thai-Kampuchean border.

RECOMMENDATION: Series of combinations of sulfonamides and dihydrofolate reductase inhibitors should be evaluated using a modification of this assay system to identify appropriate combinations of drugs which would have significant antimalarial activity against Fansidar-resistant field isolates. This system would also provide data on appropriate ratios of the combinations which show maximum potentiation. Also, this assay should be used in regions with emerging resistance to Fansidar to evaluate the extent of resistance to each component and to the combination.

The Effect of New Antimalarial Drugs on Acute Toxoplasmosis in the Hamster Model

PROBLEM: Toxoplasmosis in man is presently treated with a combination of sulfadiazine and pyrimethamine. Pyrimethamine is a teratogenic drug and can also cause severe bone marrow suppression when given at the dose required for antitoxoplasma effect. In addition, this drug combination has not been satisfactory for treating chronic active toxoplasma chorioretinitis. Recently toxoplasmosis has been found as a complication in cases of acquired immunodeficiency disease(AIDS). There is clearly a need for alternative toxoplasmosis chemotherapy. In experimental toxoplasma chorioritinitis in rabbits, clindamycin has been shown to be an effective treatment. Both clindimycin and minocycline, a tetracycline analogue, have shown some prophylactic effect on acute toxoplasmosis in mice. Presently there are several new antimalarial drugs that are being tested in man. Because antitoxoplasma drugs such as pyrimethamine and minocycline are also antimalarial, these new malaria drugs may be effective in treating toxoplasmosis.

OBJECTIVE: To determine if new antimalarial, trypanosomal, or leishmanial drugs have any effect on experimental toxoplasmosis in the hamster model.

PROGRESS: To date 11 antimalarials, 1 antibiotic and bactrin have been tested in various dose ranges.

FUTURE OBJECTIVES: Additional drugs will be tested as they become available.

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Evaluation of Plasmodium cynomolgi Sporozoite-Induced Infections of Captive Born Macaca fasicularis

PROBLEM: India has ceased exportation of rhesus monkeys which are used in the Plasmodium cynomolgi antimalarial compound testing model. A systematic evaluation of captive born Macaca fascicularis has not been completed to determine if this species could be used to supplement scarce rhesus monkeys.

PROGRESS: In previous years the captive born splenectomized cynomolgus has been shown to be capable of supplementing the rhesus in radical curative antimalarial compound testing. Fifteen AFRIMS produced splenectomized cynomolgus have been inoculated with P. cynomolgi sporozoites during FY 84 to supplement rhesus monkeys in the radical curative test. After test completion, one splenectomized cynomolgus was reinoculated with P. cynomolgi sporozoites separated by a gradient density method, and this monkey relapsed when given an inactive compound. Thus far, results using splenectomized cynomolgus indicate they are as dependable as the intact malaria naive rhesus in the radical curative test, providing thick blood films are examined by experienced personnel.

FUTURE OBJECTIVES:

1. Gather data on a larger number of splenectomized cynomolgus for significant comparison to the rhesus.

2. Use this model for evluating potentially toxic compounds.

PUBLICATIONS:

Sporozoite-Induced *Plasmodium cynomolgi* Infections in Captive Born *Macaca fascicularis.* Ward, G.S., LTC, VC, P. Hansukjariya, BSc., S. Wongsepradit, R.G. Andre, MAJ, MSC, D.E. Davidson, Col, VC. *Southeast Asia J. Trop Med Pub Hlth 15:* 12-18, 1984.

Department of veterinary of Veterinary Medicine Support

1. LABORATORY ANIMAL COLONY ACTIVITIES:

During FY 1984 the laboratory animal colony supplied a large number of research animals to both AFRIMS investigators and investigators from other research institutions, universities and hospitals. A total of 108,180 mice, 351 guinea pigs, 1552 hamsters, and 62 rabbits were supplied to AFRIMS and the following institutions: Thai Component AFRIMS, Thailand Institution of Science, Thailand Department of Livestock Development, Rajvithi Hospital, Faculty of School of Public Health, Faculty of School of Tropical Medicine (M.U.), Faculty of Pharmaceutical Science (CU), International School of Bangkok, Pesticide Research Lab, Department of Agriculture, Chulalongkorn, Faculty of School of Veterinary Medicine (CU), Laboratory Animal Research Center, Chulalongkorn Hospital and SEAMEO Tropical Medicine. Besides monkeys and the above species, 5 other species (ducks, dogs, sheep, geese, chicks and a cow) were maintained for research by AFRIMS and collaborating institutions. In addition, 60,720 ml of sheep, goose, rabbit, calf, monkey, horse and mouse blood was issued to AFRIMS, the Thai Component, Seventh Day Adventist Hospital, MU, Medical Unit US Embassy, Faculty of School of Public Health and Faculty of School of Tropical Medicine-Siriraj Hospital.

During FY 84 there were 51 rhesus and 17 cynomolgus born in the AFRIMS Primate breeding colony. In the near future all breeding cages will be filled with approximately 9 females and 1 male. There are presently 3 group breeding cages of cynomolgus and 12 for rhesus. Two new breeding cages are in the planning stages. An additional 3 group cages are used to hold weanling rhesus. Nonhuman primate production at AFRIMS will increase in FY 85 since more females and breeding cages have been added. During FY 84 there was an outbreak of *Shigella flexneri 4* in rhesus in the experimental and primate breeding colonies. A marked leukemoid reponse was noted so the potential of rhesus to mount this response to other strains of shigella was investigated. Approval was not obtained for shipment of any monkeys from AFRIMS to WRAIR. A rabies chemotherapy study in collaboration with MU was continued. A chemotheraputic study of toxoplasmosis in the hamster using new antimalarials was started. Evaluation of potential chemotherapeutic agents in animal models for cerebral malaria has been ongoing in collaboration with AFRIMS Dept Med and MU.

There were 374 CSF samples drawn and 819 sereny tests completed by Vet Med personnel. Five sheep and 4 rabbits were used in snake antivenum production in collaboration with Mahidol University. Eleven rabbits were used in Anaba and Ogawa Cholera strain antisera production for Dept of Bacteriology. Chicks were evaluated as possible replacements for sentinel pigs in testing JE virus presence but were not as dependable as pigs. Ducks were evaluated as a possible reservoirs for JE but only a low level, nonpersistant viremia developed. A JE vaccine study using 30 cynomolgus and a DHF study using 19 rhesus mothers and infants each were completed. Five retired goose blood donors and 5 rabbits were donated to the Kon Kaen Center for the Education of the Blind.

Adequate daily production of CD^1 suckling mice has continued in FY 84 as >500 suckling mice were produced daily. Useage has approximated >400 daily. This high production has been sustained by maintaining the breeding stock under virus proof, cage filter tops in 2 air conditioned rooms. Some use of BALB/c mice also produced at AFRIMS is beginning. The facility refurbishing and repainting program has been continued. New equipment has been obtained to update and replace outdated or unserviceable items.

2. HEMATOLOGY LABORATORY SECTION:

Activities of the hematology/histopathology laboratory are summarized as follows:

MALARIA:

Malaria parasite counts	8,064 thick & thin blood films
Sporozoite counts	30 slides
RBC counts	778 specimens
WBC counts	1,935 specimens
Drugs weighed for malaria project	2,633 doses
CBCs in laboratory and domestic animals	570 cases
Fecal examinations for parasites	10 specimens

LEPTOSPIROSIS:

Microagglutination screening tests	1,418
Microagglutination titrations	520
Cultures	1,147 specimens
Hamster inoculated	740

JE Virus Study:

Monkey	563	serum samples; CSF 168 samples
Pig CQ farm	203	serum samples
Lab(VM)	159	serum samples
Chicken	70	serum samples
Duck	66	serum samples

HISTOPATHOLOGY:

534 cases; 978 blocks, 3625 slides, 66 special stains, 733 H & E stains.

VM - 289 cases, 733 blocks; 2,199 slides, 733 H & E stains; 66 special stains

Virology(Johnson) - 126 cases; 126 blocks; 746 slides Brain-frozen sections)

Virology(Leake) - 119 cases; 119 blocks; 680 slides (Mosquito paraffin-sections)

PUBLICATIONS:

1. Use of Steptomycin and Isoniazid During a Tuberculosis Epizootic in a Rhesus and Cynomolgus Breeding Colony by G.S. Ward, LTC, VC, M.R. Elwell, MAJ, VC, M. Tingpalapong, DVM, LLB, J. Pomsdhit. Accepted for publication by Lab Anim Sci .

2. What's Your Diagnosis: Plasmodium knowlesi MALARIA by G.S. Ward, LTC, VC, M.r. Elwell, MAJ, VC, P. Hansukjariya, BSc. Published in LAB ANIMAL 12 No. 8): 13-14, 1983.

3. Evaluation of Inactivated Feline Panleukopenia and Parvovirus Vaccines during an Epizootic of Canine Viral Enteritis in a Dog Breeding Colony. Markpol Tingpalapong, DVM, LLB.; Michael A. Ussery, CPT; Sukhum Sujarit, LTC, RTA; Somsak Raksil, CPT, RTA; Richard E. Whitmire, LTC, VC. J. Thai Vet Med Assoc. 35: 17-27, 1984.

Evaluation of Experimental Antimalarial Drugs for Radical Curative Activity in the Rhesus Monkey

PROBLEM: Primaquine is the drug of choice for treatment of refractory, relapsing *Plasmodium vivax*, *P. malariae* and *P. ovale*. In addition to being the only drug presently in use, which raises the question of resistance, primaquine has several undesirable side effects. Nausea and methemoglobinemia, as well as hemolysis in patients with Glucose-6-Phosphate Dehydrogenase (G-6-PD) dificiency, necessitate small daily doses for 2-3 weeks. Deficiency of the enzyme G-6-PD is hereditary and common in Thailand as well as in other malaria prevalent areas such as Africa. These reasons have prompted a search for an effective but less toxic radical curative drug. Radical Curative means elimination of the persistant exoerythrocytic or liver schizonts of relapsing malaria (primarily vivax). The Rhesus - *P. cynomolgi* model is a highly reliable test system for evaluating compound efficacy against exoerythrocytic stages.[1,2,3]

OBJECTIVE: To test candidate antimalarial compounds for radical curative activity in the sporozoite induced *Plasmodium cynomolgi* - Rhesus model.

PROGRESS: Compound testing in FY 84 has been greatly faciliated by obtaining sufficient test animals for the first time since FY 79. Forty young rhesus were imported from the U.S. and 47 AFRIMS produced rhesus were tested along with 15 splenetomized cynomolgus from the AFRIMS breeding colony. This has allowed larger groups to be tested at each sporozoite inoculation and the backlog of untested compounds has thereby been eliminated. All compounds presently at AFRIMS have begun evaluation or have completed testing. The monkey-mosquito cycle has been interrupted with the last sporozoite inoculation (SP77) being made on 31 Aug 84. The cycle will be reestablshed in FY 85 when sufficient new compounds have been synthetized and mailed to AFRIMS. During FY 84, a total of 33 compounds have completed testing and 13 compounds are presently on test. The 8-aminoquinolines continue to be the main compound type showing radical curative activity.

FUTURE OBJECTIVE: To continue screening newly synthetized compounds as they become available.

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The Response of British Expatriates and Nepalis to the Biken Monovalent Japanese Encephalitis Vaccine

PROBLEM: Collaborative field studies between AFRIMS, the British Royal Army Medical College, and the London School of Hygiene and Tropical Medicine have identified a seasonal outbreak of acute encephalitis in the Terrai region of Nepal which is suspected to be due to Japanese encephalitis virus. During the summer of 1983 a vaccination programme was carried out on the personnel at the camp using the Biken monovalent vaccine. British military personnel and gurkha soldiers received three doses of the vaccine at 10 day intervals and nepali hospital staff received two doses.

OBJECTIVE: To determine the immunogenicity of the JE vaccine administered under field conditions to English and Nepali soldiers in an area threatened by epidemic encephalitis thought to be due to JE.

PROGRESS: Pre-vaccination and post vaccination sera were obtained and sent to AFRIMS for neutralisation tests, and portions of each specimen were forwarded under code to Japan for independent assessment.

The results of AFRIMS tests on 75 serum pairs against the parental Japanese virus strain Nakayama-Yoken and a local Thai strain KE094 are shown in Table 1. Of 33 British personnel negative before vaccination 30 developed detectable antibody titre against both strains. About 30% of the gurkha soldiers had pre-existing antibody to JE virus and of the remainder 78% developed antibody to the Japanese virus and 85% to the Thai virus. In the two shot hospital personnel group a much lower seroconversion ratio was noted of 36% to the Japanese virus and 31% to the Thai virus.

Comprehensive results were obtained by the Biken Institute (manufacturers of the vaccine) using 5 JE virus strains, the Japanese Nakayama-Yoken and Chinese Beijin-1 strains, and the three Thai strains, 2372, p-19Br and Chiang Mai (Table 1). Although the results confirmed the pattern obtained at AFRIMS, in general, the results obtained by the Biken Institute showed higher levels of seroconversion and there was some concern that the sensitivity of the AFRIMS test system was too low.

TABLE 1.
SEROCONVERSION RATE IN JE VACCINEES.

LABORATORY	AFRIMS		BIKEN				
TEST VIRUS	NAK-YOK.	KE094.	NAK-YOK	2372	BEIJIN	P-19Br	CHAI MAI
SUBJECTS:							
BRITISH PERSONNEL	91	91	100	97	90	90	87
GURKHA SOLDIERS	78	85	95	95	89	89	90
NEPALI STAFF	36	31	98	90	68	73	63

RECOMMENDATIONS:

1. When neutralization tests are done with the vaccine strain of virus, the vaccine provides almost complete immunization of recipients of two doses.

2. The lower response rates when other strains are tested suggests that for a universal vaccine, at least a bivalent vaccine may be necessary.

3. On the basis of these data it is reasonable to procede with studies of the protective efficacy of the BIKEN vaccine.

4. Reasons for different results between the two laboratories should be sought immediately.

5. Further tests of 1 year sera and post-booster sera will provide further valuable data for planning of larger scale vaccine field trials.

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3. Boudreau E.F., Pang L.W., Dixon K.E., Ua-Umnuay T., Phintuyothin, P. The Treatment of P. falciparum Malaria with a Combination of Quinine and Tetracycline-2nd Annual Regional Laboratory Meeting 20-24 May 1984, Cha-Am, Thailand.

4. Boudreau E.F., Pang L.W. Gem Miner's Prophylactic Study-At the 2nd Annual Regional Laboratory Meeting 20-24 May 1984, Cha-Am, Thailand.

5. Boudreau E.F. Overview of the Conduct of Antimalarial Drug Trials in Thailand at AFRIMS-At the Overseas Commander's Meeting 28-29 November 1983 National Naval Medical Center, Bethesda, Maryland.

6. Boudreau, E.F., Pang L.W., Dixon K.E., Webster H.K., Thosingha L., Phintuyothin P. and Canfield C.J. Comparable Efficacy of Halofantrine and Mefloquine in the Treatment of Drug Resistant P. falciparum Malaria on the Thai-Kampuchean Border-At the Joint meeting of The American Society of Tropical Medicine and Hygiene and the American Society of Parasitologist in San Antonio, Texas 4-8 December 1983.

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11. Webster, H.K., Boudreau, E.F., Pavanand, K., Yongvanitchit, K., and Pang, L.W. Antimalarial Drug Susceptibility Testing of *Plasmodium falciparum* in Thailand Using a Microdilution Radioisotope Method. American Journal of Tropical Medicine and Hygiene (accepted for publication).

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 0A6448	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750A	3M463750D808	AK	003 WWGG
b. [illegible]				
c. [illegible]	CARDS			

11. TITLE *(Precede with Security Classification Code)*
(U) Advanced Vaccine Development

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE 58 05	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT	
a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	3.0	728
85	3.0	855

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Div of CD&I
b. ADDRESS *(include zip code)* Washington, DC 20307-5100	b. ADDRESS Washington, DC 20307
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR BERMAN, S L
d. TELEPHONE NUMBER *(include area code)* 202-576-3551	d. TELEPHONE NUMBER *(include area code)* 301-427-5208
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* ALTIERI, P L
	g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* POWELL, C

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U)RAM I; (U)Biological products; (U)Typhoid-Shigella hybrid vaccine; (U)E.coli-Shigella hybrid vaccine; (U)E.coli vaccines; (U)Meningococcal B protein-polysaccharide vaccine; (U)Gonococcal lipopolysaccharides; (U)Bioassay; (U)Freeze-dry

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) This work unit is concerned with development of manufacturing methods and production of new vaccines for military use and with modification of existing biologicals to increase effectiveness, reduce reactivity, to afford greater stability and to minimize logistic requirements.

24. (U) Increased effectiveness and reduced reactivity are pursued by applying new physical and chemical methods to processing. Improvement in stability and reduction of logistic requirements are achieved by application of modern freeze-drying and packaging techniques.

25. (U) 83 10 - 84 09 Investigations on the development of new and improved biologicals for military use have continued. A lot of a Salmonella typhosa-Shigella hybrid and a lot of an Escherichia coli-Shigella hybrid, live, oral, freeze-dried vaccine have been prepared, tested and made available for human use. Two lots of an E. coli pilus vaccine comparing methods of inactivation and 2 lots of an E. coli whole organism vaccine have been prepared, tested, and made available for human studies. Experimental meningococcal protein-polysaccharides have been prepared comparing the effect of methods of purification on yield and immunogenicity. Agar grown harvests of 3 strains of Gonococcus have been prepared in sufficient quantities to yield for each strain 60 grams of acetone-killed and dried material for purification of the lipopolysaccharide component. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83- 30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE

PROJECT 3M463750D808 MEDICAL DEFENSE AGAINST MILITARILY IMPORTANT DISEASES

WORK UNIT 003 ADVANCED VACCINE DEVELOPMENT

Investigators:

Principal: Sanford L. Berman, Ph.D.

Associates: Patricia L. Altieri
Calvin Powell

Problems and Objectives

Investigations on the development of new and improved biological products have continued. A method of producing a genetically stable *Salmonella typhosa*-*Shigella sonnei* hybrid, live, oral vaccine was developed and the methods applied to the production of an *Escherichia coli*-*Shigella flexneri* hybrid vaccine. Work was also initiated on producing two *E. coli* pilus vaccines suitable for human use comparing two methods of inactivation. In addition, two whole culture *E. coli* vaccines from each of two strains would be produced for comparison to the pilus products. Work was continued on improving the production methods of meningococcal protein-polysaccharide vaccines in an effort to improve yield, purity and pyrogenicity. The system developed for growing and harvesting the *E. coli* organism was applied to different strains of gonococci to determine if the system would be suitable for obtaining sufficient materials for studies on the isolation of the lipopolysaccharide component of the different strains.

Progress

A lot of a *S. typhosa*-*S. sonnei* and an *E. coli*-*S. flexneri* hybrid, live, oral vaccine was produced in a stable, freeze-dried form. These were tested for genetic stability, viability, purity and safety, and are currently being evaluated in man for safety and efficacy. Two lots of an *E. coli* pilus vaccine made from the same strain of organism, but one inactivated by formalin and the other by irradiation, were prepared. In addition, two lots of an *E. coli* whole organism vaccine each made from a different strain of the organism was also prepared. The four lots were tested for sterility, purity and safety and made available for human trials. Studies continued on developing a meningococcal protein-polysaccharide more chemically defined, more potent and less reactogenic than those previously tested in man. Phenol inactivated harvests are being treated with different detergents and the resultant products are currently being evaluated both chemically and in animals. The procedures developed for the growth and harvesting of the E. coli vaccines organism were applicable to the growth and subsequent pure culture harvests of various gonococcal strains. Agar grown harvests of three strains of gonococcus have been prepared in sufficient quantities to yield for each strain 60 grams of acetone-killed and dried material for subsequent purification and isolation of the lipopolysaccharide component.

Recommendations

Other strains of S. typhosa or E. coli-Shigella hybrid have been developed and will be produced as required. In addition, if either of the hybrids currently under test in humans is successful, additional lots of these products may be required for further testing. The direction of the future work with the meningococcal vaccines will depend on the results of the current studies. If any procedure yields a promising product, a lot of this material will be produced suitable for evaluation in man. Production of agar grown harvests will continue for 3 additional strains of gonococcus. The Department of Biologics Research will continue to provide production and freeze-drying support to other investigators as required.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA OG 7009	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D· Change	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750A	3M463750D808	AP	004 WWGP
b. CONTRIBUTING				
c. XXXXXXXXX	CARDS			

11. TITLE *(Precede with Security Classification Code)*
(U) Gonococcal Vaccine Development

12. SUBJECT AREAS
0603 Biology 0613 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 06	Cont	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORK YEARS	b. FUNDS *(In thousands)*
84	3.0	306
85	5.0	316

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of CD&I
b. ADDRESS *(include zip code)* Washington, D C, 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, D C, 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Boslego, J
d. TELEPHONE NUMBER *(include area code)* (202)-576-3551	d. TELEPHONE NUMBER *(include area code)* (202)-576-3601
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* McChesney, D g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Chung, R C

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Volunteers; (U) RAMI; (U) Neisseria; (U) Gonorrhea; (U) Gonococcal Vaccine; (U) Antigen; (U) Immunity

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23(U) To develop a gonococcal vaccine. Gonorrhea has reached epidemic proportions in field troops in some areas (20 cases/1000/day) and gonococcal strains have developed resistance to penicillin as well as second line drugs.

24(U) The general approach is to study and determine the immunologic response to naturally occurring gonococcal infections, determine the gonococcal antigen(s) responsible for that immunologic response, correlate these studies with natural disease, and then develop that antigen(s) as a vaccine candidate. Gonococcal pili which function to attach the gonococcus to epithelial mucosal cells, have been isolated and purified. Antibodies directed against gonococcal pili block the attachment of gonococci to epithelial cells. A prototype gonococcal pilus vaccine has been tested in humans and has been found to be safe and immunogenic. A field trial to test the efficacy of Pgh 3-2 vaccine was conducted in troops in ROK Jan-Mar 1983. 3252 volunteers entered the study. The vaccine did not protect against gonococcal infections. Serum, secretions and strains collected during the study period will be analyzed. Second generation vaccines will be constructed utilizing multiple serotypes, a more antigenic pilus, a fragmented common determinant, or a synthetic peptide.

25(U) 83 10 - 84 09. Serum from volunteers (vaccine and placebo recipients, infected and non-infected) were analyzed by ELISA to determine class-specific antibody responses to homologous and heterologous pili. Most vaccinees had a good antibody response to Pgh 3-2 and a high titer did not appear to protect against disease. Antibody levels were lower to heterologous pili and pili from infecting strains suggesting that the vaccine did not elicit broadly cross-reacting antibodies. Antibodies in secretions are currently being evaluated. Preliminary work on second generation vaccines has begun. (For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sept 84).

Project 3M463750D808 MEDICAL DEFENSE AGAINST MILITARILY IMPORTANT DISEASES

Work Unit 004 Gonococcal Vaccine Development

Investigators:

Principals: LTC John W. Boslego, MC
LTC Raymond C. Chung, MC
Samuel B. Formal, Ph.D.

Associates: COL Jerald C. Sadoff, MC
CPT Daniel McChesney, MSC
Robert Seid, Ph.D.

Problem and Objectives

Gonorrhea is a major medical problem in the United States Armed Forces. Greater than 100,000 cases are reported each year in the military resulting in a substantial amount of man-days lost. Antibiotic resistance is increasing and threatening the easy outpatient management of gonorrhea. The current methods of control have been dismally ineffective. Despite effective antibiotic therapy and extensive contact tracing, the infection rate continues to remain high. The objective of this research is to develop a vaccine which will prevent gonococcal infection. The program also monitors and advises on the epidemiology, new diagnostic methods, changing antibiotic susceptibilities, and antibiotic treatment regimens for gonococcal infections as well as other agents of sexually transmitted diseases.

Progress

Although a prototype gonococcal vaccine consisting of purified pilus protein from a single strain was effective in preventing disease in a human challenge model, a large-scale field trial in the Republic of Korea (1983) failed to demonstrate protection. The vaccine elicited antibody responses in serum to pili from the vaccine strain and a heterologous strain which persisted through the study. These antibodies were either too low or directed at a determinant not essential for virulence. Antibody levels were also measured against pili from infecting strains and found to be cross-reactive.

Work has begun on a second generation vaccine. Three avenues are currently being pursued: 1) identification and testing of a single pilus protein which elicits a considerably higher antibody level against "common determinants" 2) combining pili from representative strains into a single vaccine to make a "mixed serotype" vaccine 3) identification and testing of synthetic peptides representing common, functional segments of the pilus protein.

Future Studies

1) Measure antibody levels in secretions.

2) Determine antibody levels against pili from additional infecting strains.

3) Pili typing - comparison of pili from infecting strains with pili from the vaccine strain.

4) Functional (IEA) assays on serum and secretions.

5) Natural disease study looking for antibody response to a variety of gonococcal antigens.

6) Challenge studies utilizing 2nd generation vaccines to look for protection against homologous and heterologous strains.

7) Confirm pili as a virulence factor in the human challenge model.

8) Field trial with promising second generation vaccine.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OG 6766
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750A	3M463750D808	AC	005 WWGQ
b. CONTRIBUTING				
c. [illegible]	CARDS			

11. TITLE *(Precede with Security Classification Code)*
(U) Role of Polysaccharide Antigens in Immunity

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE: 80 10
14. ESTIMATED COMPLETION DATE: Cont
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT/GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	2.0	470
85	2.0	486

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)*: Washington, D C 20307 5100
c. NAME OF RESPONSIBLE INDIVIDUAL: TOP, F H JR
d. TELEPHONE NUMBER *(include area code)*: (202)-576 3551

20. PERFORMING ORGANIZATION
a. NAME: Division of CD&I
b. ADDRESS:
Walter Reed Army Institute of Research
Washington, D C 20307 5100
c. NAME OF PRINCIPAL INVESTIGATOR: Formal, S B
d. TELEPHONE NUMBER *(include area code)*: (202)-576 3344
f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*: Zollinger, W
g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*: Boslego, J

21. GENERAL USE: FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) RamI; (U) vaccine; (U) volunteers; (U) meningococci

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23(U) Infectious diseases continue to be a threat to military operations. Effective vaccines are a means to control infections, and several have reached the stage of development which requires preliminary testing in human beings for safety and antigenicity. Current emphasis is on the testing of meningococcal vaccines. Preliminary safety and antigenicity studies in human beings of experimental vaccines are necessary before efficacy studies of experimental vaccines can be undertaken.

24(U) Experimental vaccines, consisting of purified products extracted from bacteria, are prepared in pilot lots by the Department of Biologic Products, WRAIR. These are tested for safety and antigenicity in the laboratory. Following review by the SGO and the Bureau of Biologics, FDA and with the consent and cooperation of Field Commanders, these vaccines are tested in soldier volunteers for safety and antigenicity.

25(U) 83 10 - 84 09 A tetravalent A, C, Y, W135 vaccine is now in routine use in Army recruits. Group B meningococcal disease remains a problem. A multivalent vaccine, lot ACYW2b15-2, which contained outer membrane proteins from two group B serotypes combined with serogroup A, C, Y, and W135 capsular polysaccharides was tested for safety and immunogenicity in 10 laboratory volunteers and then in 54 recruit volunteers. The vaccine appeared to be safe with side effects limited to mild reactions. Bactericidal antibodies against group B strains of homologous serotypes were induced in about 85 per cent of volunteers. The follow up serology is still in progress, but preliminary results indicate that a booster dose of protein will be needed and that immunity against group B disease will likely be type specific (For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 to 30 Sept 84).

DD FORM 1498 83 MAR

EDITION OF MAR 68 IS OBSOLETE.

Project 3M463750D808 MEDICAL DEFENSE AGAINST MILITARILY IMPORTANT DISEASES

Work Unit 005: Role of Polysaccharide Antigens in Immunity

Investigators:

Principals: Wendell D. Zollinger, Ph.D.
John Boslego, M.D.
Samuel B. Formal, Ph.D.

Associate: Brenda L. Brandt, M.S.

Problem

Infectious diseases continue to be a threat to military operations. Effective vaccines are a means to control infections, and several have reached the stage of development which requires preliminary testing in human beings for safety and antigenicity. Current emphasis is on the testing of meningococcal group B vaccines. Preliminary safety and antigenicity studies in human beings of experimental vaccines are necessary before efficacy studies can be undertaken. Experimental vaccines, consisting of purified products extracted from bacteria are prepared in pilot lots by the Department of Biologics Research, WRAIR. These are tested for safety and antigenicity in the laboratory. Following review by the SGO and the Office of Biologics, FDA and with the consent and cooperation of Field Commanders, these experimental products are tested in soldier volunteers for safety and antigenicity.

Progress

Six different lots of meningocccal group B vaccine differing in composition and method of preparation were produced for human use during FY83. These vaccines consisted of serotype 2b and/or serotype 15 outer membrane proteins noncovalently complexed to capsular polysaccharide. Five of the six lots contained the group B polysaccharide and the sixth the tetravalent A, C, Y, W-135 polysaccharide mixture. As a result of new data suggesting that antibodies to the group B polysaccharide were not protective and might be cross-reactive with fetal and neonatal brain tissue, only the one vaccine (lot ACYW2b15-2) that did not contain the group B polysaccharide was tested in human beings. This polyvalent vaccine contained outer membrane proteins from the group B serotypes 2b and 15 noncovalently complexed to capsular polysaccharides of groups A, C, Y, and W135. The vaccine was initially tested in 10 laboratory volunteers at the WRAIR. After obtaining informed consent, a single dose containing 300 ug of antigen (120 ug protein and 180 ug polysaccharides) was given subcutaneously in the upper arm. Reactions to the vaccine were limited to local erythema, soreness and induration at the vaccination site. No systemic reactions were observed. The polysaccharides produced an antibody response fully equivalent to that obtained with the purified polysaccharides alone, and the proteins elicited bactericidal antibodies against the group B vaccine strains in 80% of those vaccinated. The vaccine was further tested in 101 recruits at Fort Benning, GA. The experimental vaccine was given to 54 volunteers and the licensed tetravalent meningococcal vaccine to 47 who were followed as a control group. Again, a single dose of 300 ug of total antigen was given and throat cultures and blood specimens were obtained at 0, 2, 4, 6, 9, and 15 weeks. The serological analysis of the sera obtained is still in progress, but preliminary data indicate that the polysaccharide part of the experimental vaccine was fully immunogenic and that the proteins induced a four-fold or greater increase in bactericidal antibodies in about 80-90% of volunteers. The vaccine was found to be safe although local reactions were significantly greater with the experimental vaccine than with the licensed tetravalent vaccine. The specificity of the bactericidal antibody response to the proteins is still under investigation, but as observed previously, it appears that the bactericidal antibodies are effective only against group B strains of the same or related serotypes. The data also suggest that it may be necessary to give a booster dose to increase the percentage of responders and to maintain the increased titer of antibodies for a longer period of time.

Collaborative studies are under way to further test the experimental vaccine lot ACYW2b15-2 for safety and immunogenicity in human volunteers in Norway where an epidemic of group B, serotype 15 disease has been going on for several years.

Several additional studies of a developmental nature were initiated to find alternate methods for solubilizing and presenting the outer membrane proteins for use as a booster dose and to attempt to identify surface antigens other than the polysaccharide which are common to all group B strains. The first of these studies has been focused on evaluating the use of alkaline detoxified lipopolysaccharide in place of the capsular polysaccharide to complex with the proteins. Several small lots of vaccine were prepared and tested in animals with encouraging results. In the second study, monoclonal antibodies prepared against whole viable meningococci were screened for cross reactivity against a series of different group B strains. One clone was found which produces antibodies that bind to a previously unrecognized outer membrane protein which appears to be common to all N. meningitidis, N. gonorrhoeae, and N. lactamica. It has been determined that human beings have a good antibody response to this antigen as a consequence of natural infections, but it is not known if the antibodies are bactericidal. The monoclonal antibody did not have bactericidal activity.

Future plans

The serologic evaluation of specimens from the ACYW2b15-2 vaccine study will be completed. Additional human studies will be required to evaluate the timing and effectiveness of a booster dose and the safety and immunogenicity of the proteins when presented as a complex with detoxified lipopolysaccharide and/or adsorbed to alum as an adjuvant. Two new lots of vaccine will be prepared for human use to be used in these studies. One containing the same components as lot ACYW2b15-2 but essentially free of lipopolysaccharide, and the other containing the same proteins complexed to detoxified lipopolysaccharide. If a suitable population can be identified, an efficacy study needs to be done to determine if antibodies to the outer membrane proteins can provide protection. Such a population may currently exist in Norway. Efforts to identify common antigens with vaccine potential will continue. The serotype of new group B case isolates will be monitored by monoclonal antibody serotyping.

Bibliography

1. Griffiss, J. M., B. L. Brandt, D. D. Broud, D. K. Goroff, and C. J. Baker. 1984. Immune response of infants and children to disseminated infections with *Neisseria meningitidis*. J. Infect. Dis. 150:71-79.

2. Zollinger, W. D., E. E. Moran, H. Connolly, R. E. Mandrell, and B. Brandt. 1984. Monoclonal antibodies to serotype 2 and serotype 15 outer membrane proteins of *Neisseria meningitidis* and their use in serotyping. Infect. Immun. 46:260-266.

3. Zollinger, W. D., J. Boslego, E. Moran, B. Brandt, H. Collins, R. Mandrell, P. Altieri, and S. Berman. 1984. Bactericidal antibody response to a polyvalent meningococcal protein-polysaccharide vaccine. Fourth International Bacterial Vaccines Symposium, National Institutes of Health, Bethesda, Maryland.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OB 6538
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISS'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750A	3M463750D808	AG	006 WWGM
b. ~~CONTRIBUTING~~		3M162770A870		043
c. ~~CONTRIBUTING~~	CARDS			

11. TITLE *(Precede with Security Classification Code)*
(U) Characteristics of Attenuated Dengue Viruses

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE: 75 07
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT/GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	2.0	255
85	2.0	233

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)*: Washington, DC 20307 - 5100
c. NAME OF RESPONSIBLE INDIVIDUAL: TOP, F H JR
d. TELEPHONE NUMBER *(include area code)*: 202-576-3551

20. PERFORMING ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
Div of CD&I
b. ADDRESS: Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR: ECKELS, K H
d. TELEPHONE NUMBER *(include area code)*: 301-427-5208
f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*: Summers, P
g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*:

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) RAM I; (U) Attenuation; (U) Volunteers; (U)Dengue; (U) Vaccine; (U) Immunity; (U) Cell culture; (U) Lab animals; (U) Monkeys

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The objective is development, production, and assay of live-attenuated vaccines against classical strains of dengue viruses. The major types (1, 2, 3, and 4) of this virus are endemic throughout populated areas of the world, and although mortality rates are low, the incapacitation effected by these viruses and their associated sequelae could have serious impact on military time tables and troop mobility.

24. (U) Selected strains of dengue viruses are subjected to multiple passages and frequent cloning in tissue culture systems, to produce pure progeny characterized by reduced virulence and adequate antigenicity, that will serve as candidate vaccine seed viruses.

25. (U) 83 10 - 84 09 1. A small plaque, temperature sensitive clone of dengue-3 virus (clone 24/28) which is attenuated for monkeys and mice was further tested for immunogenicity in monkeys. Clone 24/28-vaccinated monkeys were challenged with 3 strains of non-attenuated dengue-3 virus. All monkeys demonstrated protection from challenge by having reduced viremias. An additional in vitro marker that distinguishes the vaccine clone from parent virus is lack of replication in human monocytes. Final safety testing of clone 24/28, including monkey neurovirulence testing, will be completed this year prior to human testing. 2. Dengue-1 and 3 virus seeds were produced in fetal rhesus monkey lung diploid cell cultures so that a source of non-attenuated virus can be safely used for vaccine efficacy tests. These seeds will also serve as reference viruses and for passage in C6/36 mosquito cells for attenuation. 3. Serum samples from a group of human subjects taken prior to dengue-2 vaccination were tested for their ability to enhance infection of human monocytes by dengue-2 virus. A sensitive microassay for enhancing antibodies was used to detect a significant higher frequency of these antibodies in yellow fever-immune individuals than in those with no previous evidence of flavivirus infection. Presence of enhancing antibodies in a recipient of the dengue-2 vaccine may be a predictor of vaccination success. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

PROJECT 3M463750D808 MEDICAL DEFENSE AGAINST MILITARILY IMPORTANT DISEASES

WORK UNIT 006 CHARACTERISTICS OF ATTENUATED DENGUE VIRUSES

Investigators:

Principal: Kenneth H. Eckels, Ph.D.

Associates: Venton R. Harrison
Doria R. Dubois
Peter L. Summers

Problems and Objectives

The project involves the development, production, and assay of live-attenuated vaccines against various strains of dengue viruses. Dengue isolates are selected from suitable sources and subjected to multiple passage and frequent cloning in cell culture systems. Pure clones of virus are screened for various markers of attenuation, including temperature sensitivity, small plaque size, lowered intracerebral virulence in mice and reduced peripheral virulence in monkeys. If the selected clones are also immunogenic in monkeys, they will serve as candidate viruses for the production of experimental seed and vaccine lots.

Progress

A dengue-3 (DEN-3) virus clone (24/28) first isolated and passaged in C6/36 cell cultures was used for the inoculation of FRhL diploid cells resulting in a lot of vaccine that could be used for human vaccination. Lot 1 of DEN-3 clone 24/28 was tested for monkey attenuation and immunogenicity. As was previously demonstrated with this clone, monkey viremia was less than that found after parent virus inoculation and immune responses as measured by neutralization and hemagglutination inhibition tests were significant. Additionally, vaccination with this clone afforded protection against three geographically-distinct strains of dengue-3 virus. Challenge with these strains four months following vaccination resulted in reduced viremias for all vaccinated monkeys indicating limited replication of the parent viruses.

In vitro markers for DEN-3 clone 24/28 include small plaque size and temperature sensitivity. Another marker that has been used to differentiate vaccine and parent (non-attenuated) viruses is replication in human monocyte cultures. A continuous monocyte line, U-937, supported replication of DEN-3 parent virus but not clone 24/28. With the addition of enhancing antibody to the cultures, both viruses replicated with the parent virus reaching higher titers. These results are similar to those found for the S-1 clone of DEN-2 virus which was shown to be attenuated for humans. Final safety testing of DEN-3 clone 24/28 Lot 1 vaccine will include neurovirulence testing in rhesus monkeys along with standard tests for adventitious microbial agents. Stability of the Lot 1 vaccine over a year in the freeze-dried state is excellent; no loss of infectivity has been observed over this period. The HSA-lactose stabilizer is superior to HSA used alone.

Virus seeds for DEN-1 and DEN-3 were prepared in FRhL diploid cells meeting the standards for a human-use product. Both of these seeds are non-attenuated viruses which can be used for efficacy testing of vaccines. Similar DEN-2 and DEN-4 seeds have already been prepared. These seeds can also be used for reference and for initiating passages in C6/36 mosquito cells for attenuation.

A DEN-1 vaccine (45AZ5) produced at the Salk Institute was used for vaccination of two human volunteers under controlled conditions. Approximately 10 days following vaccination, both volunteers exhibited symptoms of mild dengue fever. Virus isolates from serum samples of these individuals revealed that the vaccine virus had changed phenotypically and a large plaque population was evident. Re-examination of the vaccine and its master and production seeds revealed some heterogeneity of plaque size when certain lots of LLC-MK_2 cells were used for plaque assays. Clonal analysis of the vaccine and seeds revealed the presence of a high percentage of clones which were not temperature sensitive and had plaquing characteristics similar to the parent virus. Two vaccine-associated characteristics may account for appearance of clinical disease symptoms: 1) the vaccine contained temperature resistant virus which was not detected by most of the conventional plaque assays done prior to human vaccination; 2) the vaccine is unstable on introduction to humans resulting in a circulating non-attenuated virus population.

Serum samples taken from a group of yellow fever-immune (YFI) and non-immune (NI) human subjects prior to DEN-2 vaccination were tested for their ability to enhance infection of human monocytes by DEN-2 virus. A sensitive microassay for enhancing antibodies (Eab) was used to detect a significant higher frequency of these antibodies in YFI individuals than in NI subjects. Geometric mean antibody titers to DEN-2 virus after vaccination were 3-5 fold higher in YFI subjects with Eab as compared to those YFI individuals with no detectable Eab titers. In those with Eab, the titer of Eab was positively correlated with the titer of

DEN-2 antibody found after vaccination. The presence of Eab in a recipient of the DEN-2 vaccine may be a predictor of vaccination success.

Recommendations

Following safety tests of the DEN-3 clone 24/28 vaccine, preliminary human vaccine trials should begin. The recipients should be followed closely for clinical symptoms, viremia, and immune response.

Use of the DEN-1 45AZ5 vaccine should be discontinued. Work on an alternative vaccine should be initiated using several possible candidate strains. The MD-1 strain adapted to human or monkey diploid cells is one possibility. Another candidate could be produced by passage of the parent Western Pacific 1974 strain in C6/36 cells and select for an attenuated, ts population of virus.

Several DEN-4 vaccine candidates will become available through a contracting laboratory at the University of Hawaii. A PDK-53 seed has been prepared and several more will follow. A selection of the best candidate based on laboratory and animal tests will be required.

Publications

1. Scott, R. McN., Eckels, K.H., Bancroft, W.H., Summers, P.L., McCown, J.M., Anderson, J.H. and Russell, P.K. 1983. Dengue-2 vaccine: dose response in volunteers in relation to yellow fever immune status. J. Infect. Dis. 148: 1055-1060.

2. Eckels, K.H., Scott, R. McN., Bancroft, W.H., Brown, J., Dubois, D.R., Summers, P.L., Russell, P.K. and Halstead, S.B. 1984. Selection of attenuated dengue-4 viruses by serial passage in primary kidney cells V. Human response to immunization with a candidate vaccine prepared in fetal rhesus monkey lung cells. Am. J. Trop. Med. Hyg. 33: 684-689.

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4. Bancroft, W.H., Scott, R. McN., Eckels, K.H., Hoke, C.H., Jr., Simms, T.E., Jesrani, K.D.T., Summers, P.L., Dubois, D.R., Tsoulos, D. and Russell, P.K. 1984. Dengue virus type 2 vaccine: reactogenicity and immunogenicity in soldiers. J. Infect. Dis. 149: 1005-1010.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION DA 302684	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636	
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750A	3M463750D808	AI	007 WWK3
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS			

11. TITLE (Precede with Security Classification Code)
(U) Field Evaluation of prophylactic drugs and vaccines against diseases of military importance

12. SUBJECT AREAS
0613 Microbiology 0615 Clinical Pharmacology

13. START DATE 83 06	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-house

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	0.8	25
c. TYPE	d. AMOUNT	85	1.0	26
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS Division of Preventive Medicine Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Miller, R N
d. TELEPHONE NUMBER (include area code) (202) 576-3551	d. TELEPHONE NUMBER (include area code) (202) 576-3553
21. GENERAL USE FINA	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Takafuji, E T
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Kelley, P W

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Clinical Pharmacology; (U) Volunteers; (U) Biological Products; (U) Epidemiology (U) ~~RAD I~~ AMM I

23 TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To assess the suitability of certain military populations as study groups for vaccine and drug prophylaxis trials. To design epidemiologic field studies to test vaccines and drugs. To conduct field trials of prophylactic drugs and vaccines. To evaluate compliance of military groups with prophylactic regimens.

24. (U) Epidemiologic methodology is applied in the design and implementation of field trials. Studies are designed to address safety, immunogenicity, and efficacy of vaccines and prophylactic drugs directed at diseases of military importance.

25. (U) 8310-8409 With the recent implementation of the doxycycline chemoprophylaxis policy against leptospirosis at the Jungle Operations Training Center, Fort Sherman, CONUS-based military units deployed for training continue to be monitored for doxycycline compliance and illnesses suggestive of leptospirosis. Units include XVIII Airborne Corps, Special Operations Command, and Marine units. Military deployments to other geographic areas considered high risk for leptospirosis are being evaluated also to see if they would be suitable locations for future prophylaxis and therapeutic trials or places where doxycycline would also be indicated. Basic trainees at TRADOC installations are being monitored for meningococcal disease, and study populations for future vaccine field trials are being defined. The identification of new group B strains of Neisseria meningitidis in trainees last winter has necessitated a careful reassessment of multivalent vaccines.
For technical report see Walter Reed Army Institute of Research Annual Report, 1 Oct 83 - 30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

Project 3M463750D808 MEDICAL DEFENSE AGAINST MILITARILY IMPORTANT DISEASES

Work Unit 007 Field Evaluation of Prophylactic Drugs and Vaccines Against Diseases of Military Importance

Investigators.
Principal: COL Richard N. Miller, MC

Associates: LTC Ernest T. Takafuji, MC
CPT Patrick W. Kelley, MC

Objective: To assess the suitability of certain military population groups as study groups for vaccine and drug prophylaxis trials; to conduct field trials of prophylactic agesnts; to evaluate compliance of military populations with prophylactic regimens.

Progress:

1. Doxycycline Prophylaxis Against Leptospirosis. Following implementation of a policy requiring doxycycline chemoprophylaxis for U.S. forces training at the Jungle Operations Training Center in Panama during the months September-December, units were monitored for febrile illnesses suggestive of leptospirosis. This was done as a follow-up to assess compliance with the prophylaxis policy. The incidence of leptospirosis was clearly reduced in units surveyed and breakthrough cases of leptospirosis were due to non-compliance. Other geographical areas with a high prevalence of serious life-threatening infection are being assessed to see if these sites would be suitable for future prophylaxis studies directed against more virulent leptospiral serovars.

2. Acute Respiratory Disease (ARD) and Meningococcal Meningitis. ARD rates and the incidence of meningococcal infections among basic trainees are carefully monitored to identify new etiologic agents and to identify basic trainee populations that could be involved in vaccine trials.

3. Malaria Vaccine Study Populations. Military and civilian populations are being evaluated in terms of suitable populations in which to conduct efficacy trials with malaria vaccines. In collaboration with the overseas laboratories and other divisions at WRAIR, sample sizes based on predictable incidence of disease are being determined, and subpopulations who may be at greatest risk are being identified.

4. Shigella Vaccine Study Populations. In collaboration with the Department of Bacterial Diseases, populations in endemic regions of the world such as Israel are being evaluated as suitable populations for vaccine studies. The recent Brightstar exercise in Egypt clearly demonstrated the continuing threat of shigellosis and diarrheal disease to U.S. forces, but occurrence

of disease in such groups is less predictable than in indigenous populations. Therefore, civilian studies are indicated.

	DA 30 3136	84 10 01	1 DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750A	3M463750D808	AC	008 WWSE
b. ~~CONTRIBUTING~~				
c. ~~CONTRIBUTING~~	CARDS			

11. TITLE *(Precede with Security Classification Code)*
(U) Hepatitis A vaccine development

12. SUBJECT AREAS
0513 Microbiology 0603 Biology 0605 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	3.0	57
85	3.0	54

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Div of CD&I
b. ADDRESS *(include zip code)* Washington, DC 20307-5100	b. ADDRESS Washington, DC 20307 - 5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR ECKELS, K H
d. TELEPHONE NUMBER *(include area code)* 202-576-3551	d. TELEPHONE NUMBER *(include area code)* 301-427-5208
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* DUBOIS, D R g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) RAM I; (U) Volunteers; (U) Hepatitis virus; (U) Hepatitis vaccine; (U) Inactivated vaccine

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Hepatitis A virus has caused epidemic hepatitis in American soldiers in every war and is the most prevalent form of viral hepatitis that will disrupt normal functioning of a whole military unit. Presently, the only immunoprophylaxis available is temporary passive immunization with immune serum globulin. This work unit is concerned with the development of a formalin-inactivated vaccine for hepatitis A virus which will confer protective, active immunity to infection. The first lot of vaccine will be produced on a pilot scale so that it may be used for clinical safety and efficacy trials in human subjects.

24. (U) Strains of human hepatitis A virus including HM-175 will be passaged in BSC-1, MRC-5, or other acceptable vaccine substrates so that master seeds can be prepared. Suitable master seeds will be selected for further passage and vaccine preparation. Virus harvests will be subjected to formalin inactivation so that a safe, non-infectious product can be used as an immunogen in laboratory animals and man.

25. (U) 83 10 - 84 09 1. Master seeds for hepatitis A strain HM-175 were prepared in both BSC-1 and MRC-5 cells. The human diploid cell MRC-5 is more suitable for human vaccine production and will be used for future vaccine lots. A production seed and a lot of vaccine (Lot No. 2) have been prepared for HM-175 in MRC-5 cells. A single 42-day harvest of supernatant fluids and lysed cells were clarified and subjected to formalin (1:2000) at 35 degrees Centrigrade over a period of 12 days. Safety and immunogenicity tests are in progress on this lot of vaccine. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

PROJECT 3M463750D808 MEDICAL DEFENSE AGAINST MILITARILY IMPORTANT DISEASES

WORK UNIT 008 HEPATITIS A VACCINE DEVELOPMENT

Investigators:

Principal: Kenneth H. Eckels, Ph.D.

Associates: Doria R. Dubois
Peter L. Summers

Problems and Objectives

The project involves the development, production, and assay of an inactivated vaccine for hepatitis A virus. Hepatitis A strains are passaged in cells that are acceptable as substrates for the preparation of human vaccines. After adaptation, scale-up procedures are employed so that lots of vaccine can be produced for human testing. Following formalin inactivation of virus harvests, in vitro and animal tests are performed to determine antigen content and immunogenicity. Freedom from residual virus in the vaccine as well as adventitious microbial agents will be ensured by appropriate safety tests.

Progress

Hepatitis A strain HM-175 has been adapted to grow in BSC-1 and MRC-5 cells and master seeds have been prepared for this strain in both cell lines. Virus titers are adequate, ca $\geq 1 \times 10^7$ RFU/ml, for formalin inactivation and vaccine preparation. The BSC-1 cell line is a continuous cell line which is not tumorigenic. With adequate testing, the BSC-1 cell line would probably be suitable for human vaccine production. However, comparable virus titers in MRC-5 diploid cells made this the cell substrate of choice for human vaccine production. A production seed and a lot of vaccine (Lot 2) were produced in the MRC-5 cells for the HM-175 strain of hepatitis A virus. Formalin at a concentration of 1:2000 was used to inactivate approximately 3.0 liters of vaccine. Approximately half of this material will be used for immunogenicity and safety testing prior to use in man. These tests are currently in progress.

A second strain of hepatitis A virus, Clayton, was started in MRC-5 for adaptation. This strain will be held in reserve as an alternative in case HM-175 does not meet standards of potency, immunogenicity, etc. Other strains, if the need arises, could be adapted by similar techniques of passage. For adequate growth of hepatitis A strain HM-175 in MRC-5 cells, fetal bovine serum must be present in the cell culture medium. Replacement of FBS with human serum albumin is not adequate for optimal growth of the virus. This is not the case for the same virus in BSC-1 cells where optimal growth is obtained without any serum additive (L. Binn, personal communication).

Recommendations

Production techniques for preparation of hepatitis A vaccine must be refined without loss of virus titer and volume. Methods for shortening the incubation period in cell cultures (currently 42 days), multiple harvesting, removal of FBS from the product, and concentration of the product are areas being examined. The current lot of vaccine will be followed for potency and tested thoroughly for safety so that human vaccination can follow.

Publications

1. Binn, L.N., Eckels, K.H., Lemon, S.M., Marchwicki, R.H., Dubois, D.R., and Berman, S.L. Inactivated hepatitis A virus vaccine of cell culture origin. Invention disclosure submitted August, 1984.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 303137	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISS'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750A	3M463750D808	AA	009 WWGK
b. CONTRIBUTING				
c. XXXXXXXXXX	CARDS			

11. TITLE (Precede with Security Classification Code)
(U) Shigella Vaccines

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE 82 10	14. ESTIMATED COMPLETION DATE Cont	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	1.0	241
c. TYPE	d. AMOUNT	85	1.0	259
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of CD&I
b. ADDRESS (include zip code) Washington, D C 20307 5100	b. ADDRESS Walter Reed Army Institute of Research Washington, D C 20307 5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Formal, S B
d. TELEPHONE NUMBER (include area code) (202)-576 3551	d. TELEPHONE NUMBER (include area code) (202)-576 3344
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) attenuated vaccines; (U) volunteers; (U) shigella; (U) salmonella; (U) RamI; (U) monkeys; (U) rabbits; (U) mice

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23(U) Infectious diarrheal diseases continue to be a threat to military operations. Effective vaccines are a means to control some of these infections. Experimental products require safety testing in volunteers and field tests for efficacy. Current emphasis is on a preparation designed to protect against both typhoid fever and dysentery caused by Shigella sonnei and on E. coli - S. flexneri hybrid vaccines.

24(U) Experimental oral vaccines consisting of living attenuated bacteria are prepared in pilot lots by the Department of Biologics Research Department, WRAIR. These are tested for safety and antigenicity in the laboratory. Following review by the Surgeon General's Office and the Bureau of Biologics, FDA, these products are tested in volunteers for safety and antigenicity. Upon successful completion of these studies commercially prepared lots are tested for efficacy in suitable populations.

25(U) 83 10-84 09 A second efficay test of the Salmonella typhi-Shigella sonnei vaccine gave evidence of protection in volunteers. A similar product constructed at WRAIR but manufactured in Switzerland failed to protect volunteers. A second efficacy test in monkeys of the E. coli, S. flexneri 2a vaccine gave significant protection. An IND for phase I testing of this vaccine has been obtained. (For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 to 30 Sept 84).

Project 3M463750D808 MEDICAL DEFENSE AGAINST MILITARILY IMPORTANT DISEASES

Work Unit 009: Shigella Vaccines

Investigators:

Principal: Samuel B. Formal, Ph.D.

Associate: Edmund C. Tramont, M.D.

Problem

Shigellosis continues to be a military problem. Parenterally administered vaccines are not effective. Evidence has accumulated which indicate that oral immunization can be effective. The purpose of this work unit is to develop living attenuated oral shigella vaccines.

Progress

A second test in monkeys was conducted to assay the efficacy of the *E*. *coli* K-12-*S*. *flexneri* 2a vaccine. Three doses of 1×10^9 cells conferred a significant degree of protection.

Additional *E*. *coli* K-12 - Shigella candidate vaccines have been constructed. These are designed to protect against infections with *S*. *dysenteriae* 1, *S*. *flexneri* 1 and *S*. *flexneri* 3.

Bibliography

1. Tramont, E.C., R. Chung, S. Berman, D. Keren, C. Kapfer and S.B. Formal. 1984. Safety and antigenicity of Typhoid-*Shigella* *sonnei* vaccine (strain 5076-1C). J. Infect. Dis., *149*:133-136.

2. Formal, S.B. and M.M. Levine. 1984. Shigellosis 167-186 in Bacterial Vaccines. Ed., R. Germanier Academic Press, Inc., Orlando, Fl., 32887.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 30 5986	2. DATE OF SUMMARY 84 10 01	*REPORT CONTROL SYMBOL* *DD-DR&E(AR) 636*

3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750A	3M463750D808	A1	011 WWSI
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CARDS			

11. TITLE *(Precede with Security Classification Code)*
(U) Hepatitis Vaccine Testing

12. SUBJECT AREAS
0613 Microbiology 0603 Biology 0605 Clinical Medicine

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE Cont	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT	
a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	0.0	00
85	2.0	61

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of Communicable Diseases and Immunology
b. ADDRESS *(include zip code)* Washington, DC 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR BURKE, D S
d. TELEPHONE NUMBER *(include area code)* (202) 576-3551	d. TELEPHONE NUMBER *(include area code)* (202) 576-3757
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* REDFIELD, R g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* SJOGREN, M H

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Viruses; (U) Hepatitis; (U) Vaccine; (U) Volunteers; (U) RAMI

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23 (U) To evaluate the safety, reactogenicity, immunogenicity, and protective efficacy of investigational hepatitis vaccines in man, so that disability due to viral hepatitis among military personnel can be reduced.

24 (U) Hepatitis vaccines which have been approved as investigational new drugs are to be administered to human volunteers who are monitored for adverse reactions by physical examinations and clinical laboratory tests. Blood specimens and other clinical specimens are tested for evidence of viral replication and for evidence of a virus specific immune response.

25 (U) None.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 306034	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636

3. DATE PREV SUMMARY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63750A	3M463750D808	DC	012
b. CONTRIBUTING				
c. XXXXXXXXXX	CARDS			

11. TITLE (Precede with Security Classification Code)
(U) RAPID DIAGNOSIS OF DENGUE VIRUS INFECTIONS USING NUCLEIC ACID HYBRIDIZATION

12. SUBJECT AREAS
0613 MICROBIOLOGY 0603 Biology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. IN-HOUSE

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORK YEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	0.0	00
c. TYPE	d. AMOUNT	85	1.0	40
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME WALTER REED ARMY INSTITUTE OF RESEARCH	a. NAME US ARMY MEDICAL COMPONENT, AFRIMS.
b. ADDRESS (Include zip code) WASHINGTON, D.C. 20307-5100	b. ADDRESS BANGKOK, THAILAND
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR HENCHAL, E A
d. TELEPHONE NUMBER (Include area code) (202) 576-3551	d. TELEPHONE NUMBER (Include area code) 66-2-281-7776
21. GENERAL USE FINA	e. NAME OF ASSOCIATE INVESTIGATOR (If available) HOKE, C H
MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (If available) NISALAK, A

22. KEYWORDS (Precede EACH with Security Classification Code) (U) RAM I
(U) DENGUE; (U) RAPID VIRUS DIAGNOSIS; (U) NUCLEIC ACID HYBRIDIZATION

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The technical objective is to apply complimentary DNA (cDNA) probes in nucleic acid hybridization assays to detect dengue virus or dengue virus-specific RNA in clinical specimens (leukocytes or whole blood) for rapid virus diagnosis of military importance.

24. (U) This approach requires the screening of existing cDNA probes for suitability, development of clinically applicable tests, and comprehensive comparisons with current technology.

25. (U) cDNA probes suitable for use in nucleic acid hybridization assays have recently been produced under army contract. However, no attempts have been made to apply these reagents to clinical samples for the purpose of rapid viral diagnosis. Recent results have already identified a 1.2KB cDNA fragment which is dengue-2 specific. Evaluations of other probes for dengue complex specific probes will be conducted. Alteration of existing techniques to clinically appropriate assays will be performed. Concurrent identifications using serological methods and mosquito cell or mosquito isolations will be performed.

DD FORM 83 MAR 1498

EDITION OF MAR 68 IS OBSOLETE

PROJECT 3S464758D849

MEDICAL DEFENSE AGAINST MILITARILY IMPORTANT DISEASES

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION DA 3059817	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	64758	354647580849	AK	041 WWSH
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CARDS			

11. TITLE (Precede with Security Classification Code)
(U) Vaccine Development/Malaria

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	0.0	00
c. TYPE	d. AMOUNT	85	2.0	278
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Div of CD&I
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Hockmeyer, W T
d. TELEPHONE NUMBER (include area code) 202-576-3551	d. TELEPHONE NUMBER (include area code) 202-576-3544
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Weber, J g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Vaccine; (U) Antigens; (U) Malaria; (U) Protozoa; (U) DNA; (U) RAMI

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23 (U) The objective is to produce a safe, efficacious sporozoite vaccine against Plasmodium malaria which poses a significant threat to military operations in endemic areas.

24 (U) The approach used for these studies is to examine the immunogenicity of recombinant DNA produced CS protein or synthetic peptides delivered alone, with adjuvants or by incorporation of the CS gene into viral or bacterial genome.

25 (U) None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA315988	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY	64758A	3S464758D849	AA	042	WWSK
b. CONTRIBUTING					
c. XXXXXXXXXXX					

11. TITLE *(Precede with Security Classification Code)*
(U) Vaccine Development/Shigella

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		84	0.0	00
c. TYPE	d. AMOUNT	85	2.0	253
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of CD&I
b. ADDRESS *(include zip code)* Washington, D C 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, D C 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Formal, S B
d. TELEPHONE NUMBER *(include area code)* 202-576-3551	d. TELEPHONE NUMBER *(include area code)* 202-576-3344
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Shigella Vaccines; (U) Volunteers (U) RAM I (U) Monkeys (U)Bacillary Dysentry (U) Lab Animals

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23 (U) Bacillary dysentery is a continuing problem of troops operating in the field. It is necessary to be able to protect against the most prevalent serotypes.

24 (U) Candidate shigella vaccine strains constructed in the laboratory and shown to be safe for human beings and protective for either volunteers or monkeys, will be tested for efficacy under field conditions.

25 (U) None.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION DA 303989	2. DATE OF SUMMARY 84 10 01	*REPORT CONTROL SYMBOL* *DD-DR&E(AR) 636*	

3. DATE PREV SUM'RY	4. KIND OF SUMMARY A.NEW	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	64758A	3S464758D849	A5	043 WWIL
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE *(Precede with Security Classification Code)*
(U) Development of Vaccines for Enterotoxigenic E. coli

12. SUBJECT AREAS
0613 Microbiology 0611 Life Support 0603 Biology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	0.0	00
85	2.0	422

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)*
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H Jr
d. TELEPHONE NUMBER *(include area code)*
(202) 576-3551

20. PERFORMING ORGANIZATION
a. NAME Division of Medicine
Walter Reed Army Institute of Research
b. ADDRESS
Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
BOEDEKER, E C
d. TELEPHONE NUMBER *(include area code)*
(202) 576-1493
f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
ANDREWS, J
g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
REID, R H

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) vaccines; (U) adjuvants; (U) enterotoxigenic E. coli; (U) diarrhea; (U) colonization factor antigens (U)Lab Animals(U)Rabbits(U)RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Enterotoxigenic Escherichia coli (ETEC) are the major cause of Traveler's diarrhea and result in a high incidence of disabling gastrointestinal infections within the first 10-12 days in individuals entering endemic areas. The virulence mechanisms of these organisms include the elaboration of toxins (heat labile and heat stable) and of colonization factor antigens. Our objective will be to develop vaccines based on these known virulence factors to provide a protective IgA response in intestine. This research is of military importance

24. (U) Prototype vaccines will be produced and evaluated in animal systems (the rabbit) utilizing preparations of colonization factor antigen/II (including CS1 and CS3 components) and immunologic adjuvants (including muramyl dipeptide MDP and MDP covalently bound to the GM1 binding peptide fragment of cholera toxin B subunit as well as pilus components known to bind to intestinal M cells). Immunogenicity of the native CFA/II will be compared to that in the presence of adjuvants. Studies will be performed to determine the survival of CFA/II in intact intestine and methods for delivery of antigen (encapsulization) to the mucosal immune system will be tested.

25. (U) None.

DD FORM 1498 83 MAR EDITION OF MAR 68 IS OBSOLETE.

☆U.S. G.P.O. 1984-421-646/170C1

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 305994	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636

3. DATE PREV SUMMARY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY		3S464757D849		044
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE *(Precede with Security Classification Code)*
(U) Testing of Efficacy of Japanese Encephalitis Vaccine in Thailand

12. SUBJECT AREAS
0603 Biology, 0605 Clinical Medicine, 0613 Microbiology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C, In House

17. CONTRACT/GRANT

a. DATE EFFECTIVE | EXPIRATION

b. CONTRACT/GRANT NUMBER

c. TYPE | d. AMOUNT

e. KIND OF AWARD | f. CUM / TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORK YEARS	b. FUNDS *(In thousands)*
84	0.0	00
85	7.0	

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME US Army Medical Component, AFRIMS
b. ADDRESS *(include zip code)* Washington, D.C. 20307-5100	b. ADDRESS Bangkok, Thailand
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR HOKE, C H
d. TELEPHONE NUMBER *(include area code)* 202 576-3551	d. TELEPHONE NUMBER *(include area code)* 66-2 282 8141 x 291
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	e. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Nisalak, A f. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Suchard, J

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Japanese Encephalitis; (U) Vaccine; (U) Field Testing (U) Volunteers (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Determine the Efficacy of BIKEN Japanese encephalitis vaccine in preventing Japanese Encephalitis. This research is of military importance.

24. (U) The approach will be to obtain volunteers in villages in a Northern Thai province, and randomly assign them to receive monovalent vaccine, bivalent vaccine, or tetanus toxoid. Surveilance will be carried out to detect all cases of neurological disease following the immunization. Data will be stored in computers. The vaccine will be given by jet gun. Diagnoses will be made using the JE IgM capture ELISA developed in this laboratory. Data will be analyzed to determine the protective efficacy of the vaccine and the side effects associated with the vaccine.

25. (U) Progress: The protocol has been approved by the Thai Ministry of Public Health Scientific and Human Use Committee. WRAIR Scientific approval has been obtained, human use approval is pending. A protocol to evaluate the immumogenicity of JE vaccine administered by jet gun has been written, approved, and executed, demonstrating the feasibility of using the jet gun for mass inoculations of JE vaccine. Planning for the field project is in progress.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE

PROJECT NUMBER: 3S464758D849 MEDICAL DEFENSE AGAINST MILITARILY IMPORTANT DISEASES

Work Unit Number 044 : **Testing of Efficacy of Japanese Encephalitis Vaccine in Thailand**

Investigator: LTC Charles H. Hoke

Inoculation of Japanese encephalitis (JE) Vaccine Using the Jetgun. An Acceptable Method for Mass Immunization.

PROBLEM: Large scale immunization is greatly facilitated by the use of jet injector guns which can immunize many thousands of persons per day. A large efficacy study of Japanese encephalitis vaccine in northern Thailand is being planned. Jet guns of the type proposed for this study are used widely in the US military and have been used with a number of vaccines in civilian immunization programs. For immunization of children in the refugee camps in Thailand, jet guns are greatly preferred over needles. It was felt adviseable to compare the jetgun with the needle route of immunization specifically for JE vaccine. If satisfactory from an immunological point of view, it would be preferable to use jet guns for this study.

OBJECTIVE: This study was designed to determine the immunogenicity of Japanese encephalitis vaccine administered by the needle and jetgun route.

PROGRESS: In order to determine whether jetgun administration of JE vaccine produced results comparable to administration by needle, a comparative trial of immunogenicity by both routes was done. In May 1984, 2 doses of BIKEN monovalent Japanese encephalitis vaccine was administered to 41 employees of the US consulate in Chiangmai, Thailand, of whom 36 completed the study. No serious side effects occured. Sera were collected before immunization and 4 weeks after the first dose. Vaccine doses were given 1 week apart. Sera were tested under code for the presence of anti JE neutralizing antibody by the plaque reduction neutralization method.

Of 15 seronegative needle recipients, all (100%) developed an antibody titer of >=20, while 10 of 12 jet gun recipients (83%) ($p>.05$) developed titers. Overall, the needle and jetgun routes of vaccine administration produced similar excellent results, when neutralizing antibody was studied. IgM levels in postimmunization sera, while elevated above control levels, were not elevated enough to interfere with interprepation of the JE MACELISA diagnostic test.

PRNT50: NEUTRALIZING ANTIBODY IN VACCINE RECIPIENTS

Pre-immunization Titers*

Total	<10	10-40	>40-80	>80-100	>100-200	>200-400	>400	
Needle	15	2	0	2				19
Gun	12	0	1	4				17
Totals	27	2	1	6				36

Post immunization titers *

Needle	0	0	3	2	1	4	9	19
Gun	2	2	3	2	1	0	7	17
	2	2	6	4	2	4	16	36

* (Homologous neutralization titers using the Nakayama-yoken strain.)

RECOMMENDATION: The jetgun route is satisfactorily immunogenic for use in administering JE vaccine.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 306620	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636

3. DATE PREV SUMMARY	4. KIND OF SUMMARY A New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	64758A	3S464758D849	AF	045
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CARDS			

11. TITLE (Precede with Security Classification Code)
Determination of Etiology and Epidemiology of Epidemic Encephalitis in Nepal

12. SUBJECT AREAS
0603 Biology, 0613 Microbiology, 0605 Clinical Medicine

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORK YEARS	b. FUNDS (In thousands)
84	0.0	00
85	5.0	55

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME US Army Medical Component, AFRIMS
b. ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS APO San Francisco 96346-5000 (Bangkok, Thailand)
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR HOKE, C H
d. TELEPHONE NUMBER (include area code) 202 576-3551	d. TELEPHONE NUMBER (include area code) 662 282-8141 x 291
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	e. NAME OF ASSOCIATE INVESTIGATOR (If available) Leake, C NISALAK, A HENCHAL, E A f. NAME OF ASSOCIATE INVESTIGATOR (If available)

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Japanese Encephalitis; (U) Epidemiology; (U) Threat Assessment

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The technical objective is to determine the etiology of epidemic encephalitis occuring along the southern border of Nepal. This research is of military importance.

24. (U) The approach will be to send an individual to Nepal in advance of the expected outbreak equipped to collect specimens from hospital, from mosquito populations and sentinel pigs in the region of the epidemic. Specimens will be carried or shipped to AFRIMS for virus isolation and antibody testing.

25. (U) Progress to date: In coordination with the British Army Medical Department, a field team has visited Nepal on four occasions. Numerous specimens have been collected for analysis, Epidemiological and ecological studies have been performed. Laboratory analysis is presently underway.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE

PROJECT: 3S464758D849 MEDICAL DEFENSE AGAINST MILITARILY IMPORTANT DISEASES

Work Unit 045 **Determination of Etiology and Epidemiology of Epidemic Encephalitis in Nepal**

Principal Investigator: LTC Charles H. Hoke, MC

Investigations Into the Epidemiology and Etiology of Acute Encephalitis in Nepal

PROBLEM: Epidemiological data gathered at the British Military Hospital at the Headquarters of the British Gurkhas at Dharan, East Nepal, and the Koshi Zonal hospital at the town of Biratnagar some thirty miles to the south, showed that since 1977 a pattern of seasonal acute encephalitis has emerged. Case numbers ranged from a low of 64 in 1979 up to 185 in 1982 with sharp epidemic peaks during the late monsoon. Out of a total of 608 cases recorded throughout 1977 and 1982, 181 were recorded in the Nepali month of Bhadra (August 14-September13), 208 in Aswin (September14-October 13) and 97 in Kartik (October 14-November 13), with the nadir of cases (7) being in Pough (December 14-January 13). Overall fatalities were 30.36% with a slight preponderance of male cases (56.55%) and an age distribution that was fairly broadly spread with 14% in the 1-4 year group, 38% in the 5-24 year group, 19% at age 15-24 and 12% at age 25-35. This pattern was confirmed in consultations with the Nepali health authorities who have similar data on viral encephalitis cases across the whole of the Terrai rice-growing region bordering with India.

The accumulated data favoured the hypothesis that the viral agent was mosquito-borne, and in view of the recent outbreaks of Japanese encephalitis virus epidemics in the northern states of India this flavivirus was strongly suspected as the etiological agent.

OBJECTIVE: To determine the etiology and epidemiology of epidemic encephalitis in Nepal.

PROGRESS: A collaborative effort supported by the Royal Army Medical College and the London School of Tropical Medicine and Hygiene, the US Army Research and Development Command and the government of the Kingdom of Nepal was agreed upon. Preliminary visits by entomological teams have been undertaken. Definitive visits for field collecions have been made. Processing of specimens at AFRIMS Department of Virology is in progress.

Serum samples were analysed from a variety of domestic animals maintained on the camp farm and indicated high levels of flavivirus HAI activity. 18 species of mosquitoes were collected in the area and of these *Culex fuscocephalus*, *Culex gelidus*, *Culex bitaeniorhynchus*, *Culex vishnui* and *Anopheles hyrcanus* have all been implicated as Japanese encephalitis virus vectors.

Neutralisation tests performed on sera taken from personnel shortly before JE vaccination indicated that none of the British expatriates had previous exposure to the virus whereas 30% of the Nepali-borne personnel had had previous exposure. Post vaccination sera and subsequent pre and post booster sera are under analysis at AFRIMS and at the Biken Institute in Japan.

A detailed field project with the aim of isolating the etiologic agent from the area during 1984 and 1985 is currently being co-ordinated between AFRIMS, the British Royal Army Medical College and the London School of Hygiene and Tropical Medicine.

RECOMMENDATION: This study represents a multinational collaborative project that has been initiated at the highest levels within the Research and Development commands of the Armies of the UK and the USA and the Ministry of Health of Nepal. The response to the investigation in Nepal has been most enthusiastic. The study should be pursued until the etiology of epidemic encephalitis in Nepal is established unequivocally. Aid programs for controlling the disease should then be considered. This study is a prototype for studies which are essential to determine the true risk of JE throughout Asia. In Sri Lanka, for example, large epidemics of encephalitis occur each year which also may be due to JEV.

PROJECT 3M162770A870

MEDICAL DEFENSE AGAINST INFECTIOUS DISEASE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION DA0G 1295	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX
9. LEVEL OF SUM A. WORK UNIT					

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY	62770A	3M162770A870	AH	041	WWG6
b. CONTRIBUTING					
c. [illegible]	STOG 82/83 →	6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Identification of Trypanosoma rhodesiense Protective Antigens

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE 80 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	335
85	2.0	362

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of CD&I
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Hockmeyer, W T
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-3544
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Esser, K
	g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Lab Animals; (U) Mice; (U) RAMI; (U) Recombinant (U) Vaccine; (U) Trypanosomiasis; (U) Monoclonal antibody; (U) Antigen; (U) Synthetic Peptide

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) African sleeping sickness, a potential threat to military operations in Africa, has reached epidemic proportions in some areas. Currently no prophylaxis is available and the chemotherapeutic agents are toxic. The objective of the current work is to identify protective antigens of Trypanosoma rhodesiense. This work will be the basis for vaccine development.

24. (U) These studies employ monoclonal antibodies (MAbs) and an animal model to identify target antigens of protective immunity against both infective insect stage and blood stage trypanosomes. MAbs which block infections are used as markers to isolate specific antigens and for antigenic analysis of parasites from the field.

25. (U) 83 10-84 09 Mice were protected against metacyclic infection by monoclonal antibodies. These MAbs identified 17 antigenically distinct metacyclic (insect) forms of T. rhodesiense. Genes coding for three metacyclic antigens were sequenced and expressed in E. coli Immunogenicity trials with these recombinant proteins and with synthetic peptides are underway. Over 20 MAbs have been made to synthetic peptides corresponding to constant regions of trypanosome variable surface antigen. Ability of these MAbs to neutralize trypanosome infectivity is being tested. Experimental infections have been serodiagnosed by detection of circulating trypanosome antigens with MAbs. Field application of the assay system for early diagnosis of human infections is planned. For technical report see Walter Reed Army Institute of Research Annual Report, 1 Oct 83 - 30 Sep 84.

PROJECT 3M162770A870 MEDICAL DEFENSE AGAINST INFECTIOUS DISEASE

Work Unit 041 Identification of *Trypanosoma rhodesiense* Protective Antigens

Investigators:

Principals: Dr. Klaus M. Esser
LTC Wayne T. Hockmeyer, MSC

Associates: Dr. Bruce T. Hall
CPT Donald E. Burgess, MSC
Mr. Maurice J. Schoenbechler
Mr. William L. Bowie
SP5 Margaret Meadows

Problem and Objective:

African trypanosomiasis poses significant health hazards to troops operating in endemic areas. This is a progressive, generally fatal disease transmitted by the bite of infected tsetse flies. The fly vector and the causative protozoan parasite are prevalent throughout 30% of Africa. The current level of reported human disease is low primarily because of restricted land use patterns and vector control measures in highly populated areas. However, the potential of this disease is evident from previous epidemics in which 20 - 30% of the population in some areas died. Although data on the risk of infection for military troops deployed in endemic areas is not available, a high incidence of infection would be expected. Currently no prophylaxis is available and therapeutic drugs are toxic. Vaccination against African trypanosomiasis is theoretically possible. Protection against infection with a single trypanosome antigen type can easily be achieved by immunization with attenuated parasites or purified antigens. However, multiple antigen types of the parasite are present in the fly vector and a large number arise by antigenic variation in the host. The objective of this work unit is the identification and isolation of the antigens which can elicit a protective immune response against the infective insect stage (metacyclic) of the parasite.

Progress:

Protection against metacyclic-stage trypanosome infection has been achieved with monoclonal antibodies (MAbs). A pool of 16 MAbs, each specific for a different trypanosome surface antigen abolished infectivity of metacyclics from tsetse flies. These MAbs have been used in efforts to clone genes which code for metacyclic antigens in collaboration with Dr. John Donelson at the University of Iowa. Two genes have been cloned, sequenced and expressed in an E. coli system. At least one of these expressed products contains trypanosome surface antigen epitopes important for vaccine development as shown by reaction in a Western blot with MAbs able to neutralize metacyclic infectivity. Preliminary attempts to immunize animals with crude E. coli products or SDS-gel electrophoresis purified proteins failed. However, the expressed trypanosome proteins are now being separated from other bacterial products by non-denaturing methods to allow recovery of immunogenic material. Animal immunizations will be continued to determine the efficacy of E. coli-expressed trypanosome antigens.

Studies have been continued to determine if metacyclic trypanosomes in a given endemic area are antigenically stable over time. Trypanosomes isolated from six different naturally infected humans in the Lambwe Valley, Kenya over the period 1974 - 1981 were used to infect laboratory-reared tsetse flies. Infective metacyclics which developed in these flies appear to fall into three partially distinct antigen groups. At least one, and possibly all, of these groups were stable over the eight year study period. Complete immunological crossreactivity was seen between metacyclics derived from 1974 and 1981 trypanosome isolates. Both MAb analysis and infectivity neutralization studies with antisera showed that the same metacyclic antigen types developed in flies infected with these isolates. This apparent lack of "antigenic drift" with time suggests that polyvalent immunization directed against the metacyclic stage may be possible.

An alternative to polyvalent vaccination directed against different metacyclic antigen types, is development of a vaccine targeted to antigenic determinants shared among different trypanosome antigen types. Two approaches to this possibility have been pursued. Eight synthetic peptides corresponding to five regions of highly conserved amino acid sequences of the trypanosome variable surface glycoproteins (VSGs) have been produced and are being used to generate monoclonal antibodies. These MAbs are being tested for their ability to cross-react with multiple trypanosome VSGs by immunofluorescent staining of acetone fixed trypanosomes, binding to purified VSG antigens by ELISA and by neutralization of trypanosome infectivity *in vivo*. Over 20 MAbs have been produced to five of the peptides; however, no neutralizing activity has been demonstrated. Efforts to produce MAbs with different epitope specificities to these five peptides, as well as MAbs to the remaining three peptides, are continuing. Alternatively, identification of surface protein molecules which are shared among different trypanosome antigen types is being attempted. A total of 20 MAbs directed against common antigens of *T. b. rhodesiense* have been screened and two have proven to be specific for *Trypanosoma* species. These two MAbs bind to a low molecular weight (22,000 dalton) doublet present in whole antigen extracts of several distinct *T. b. rhodesiense* VATs. This antigen is surface-associated, contains protein and appears to be distinct from VSG. If MAbs specific for this antigen can protect against trypanosome infection or if immunization with the isolated target antigen is protective, then vaccine development will proceed along these lines.

Genus-specific MAbs to common antigens have also been used to develop an ELISA which detects trypanosome antigens present in sera of infected animals and humans. Seroconversion was detected by Day 12 of trypanosome infection in two rhesus monkeys. This ELISA is specific for trypanosome antigens, giving no reaction with sera from cutaneous leishmaniasis or *P. falciparum* malaria human patients. Thus MAbs directed against common trypanosome antigens show considerable promise as reagents for diagnosis of trypanosomiasis in the early, easily treatable, stage.

Recommendations:

In view of the finding that metacyclic heterogeneity appears to be restricted and that experimental immunization is possible, further work is indicated for the identification of antigens involved in eliciting a broad-spectrum immunity. Also, continued analysis of metacyclics from a range of different trypanosome isolates is necessary to determine the degree of metacyclic heterogeneity in a particular endemic area. Direct analysis of metacyclics present in tsetse flies in endemic areas is essential to allow confirmation of key laboratory findings. Monoclonal antibodies will continue to be the major tool for these studies. Production of MAbs to synthetic peptides corresponding to conserved regions of the VSG antigen should be continued in order to determine the feasibility of using peptides to induce cross-reacting immunity. This work will be relevant for trypanosomiasis vaccine development and also for establishing critical groundwork for production of synthetic vaccines in general. Efforts should be expanded on the search for invariant trypanosome surface antigens which are potential targets for protective, cross-variant immunity. In addition to conserved peptide sequences on VSG, analysis to detect other accessible protein, carbohydrate or lipid molecules should be aggressively pursued. Field testing of serodiagnostic techniques is also needed to develop clinically useful levels of sensitivity and specificity.

Presentations:

1. Systematic identification of Trypanosoma brucei rhodesiense metacyclic variable antigen types (M-VATs) important for vaccine development. K. M. Esser. WHO Workshop on African Trypanosomes. New Haven Connecticut, Nov. 1983.

2. Monoclonal antibodies against common antigenic determinants on Trypanosoma brucei rhodesiense: genus specific markers. D. E. Burgess and K. M. Esser. Am. Soc. Trop. Med. Hyg., December, 1983.

3. African trypanosomes: microbial transvestites. K. M. Esser. Parasitic disease seminar, Uniformed Services University of Health Sciences. April, 1984.

4. Molecular and immunologic studies on metacyclic antigens of African Trypanosomes. K. M. Esser. Molecular Aspects of Parasitology Symposium, U. of Pennsylvania. May, 1984.

Publications:

1. Topological mapping of protective and non-protective epitopes on the variant surface glycoprotein of the WRATat 1 clone of Trypanosoma brucei rhodesiense. T. Hall and K. Esser. J. Immunol. 130:2059-2063. (1984).

2. Variable antigen type (VAT) composition of Trypanosoma brucei rhodesiense: Discrepancy between results obtained using VAT-specific monoclonal antibodies and rabbit antisera. D.E. Burgess, K.M. Esser and B.T. Wellde. J. Am. Soc. Trop. Med. Hygiene. (in press).

3. Characterization of the genes specifying two metacyclic variable antigens in Trypanosoma brucei rhodesiense. M. J. Lenardo, A. C. Rice-Ficht, G. Kelley, K. M. Esser and J. E. Donelson. PNAS (in press).

4. Simultaneous expression of two distinct variable surface glycoproteins on individual African trypanosomes during antigenic switching. K. M. Esser and M. J. Schoenbechler (manuscript submitted).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION DA OG 6764	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636	

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISS'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A870	AO	042 WWGW
b. ~~CONTRIBUTING~~				
c. XXXXXXXXXX	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Biosystematics of Arthropods of Military Medical Importance

12 SUBJECT AREAS
0603 Biology 0606 Environmental Biology

13 START DATE 80 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	4.0	472
85	3.0	456

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Div of CD&I Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Harrison, B A
d. TELEPHONE NUMBER (include area code) 202-576-3551	d. TELEPHONE NUMBER (include area code) 202-357-1856
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Harbach, R E g. NAME OF ASSOCIATE INVESTIGATOR (if available) Peyton, E L

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Biosystematics; (U) Disease Vectors; (U) Arthropods (U) Mosquitoes; (U) Epidemiology; (U) Malaria; (U) Arboviruses (U) RAMI

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Conduct biosystematic research of important arthropod groups in support of epidemiological studies and disease control strategies of importance to the military. Disease vector groups currently under investigation are (1) Anopheles of Orient and Middle East (malaria) (2) Culex (Culex) of Middle East (arboviruses and filariasis), (3) Aedes (Stegomyia) Afrotropical Region (arboviruses) and (4) Trichoprosopon of the Neotropics (arboviruses). Build a computer data base from over 1,000,000 mosquito specimens and collection records in the Smithsonian Institution.

24. (U) Comparative morphological study of medically important mosquito groups in regions of military interest, with biological, cytogenetic, electrophoretic and cross-mating studies of vector populations, and correlation of all data to provide (1) descriptions and illustrations of species, (2) development of effective identification keys, and (3) information about medical importance of the species. File biological data for museum mosquito specimens in SELGEM computer program to facilitate biosystematic research and to understand vector behavioral patterns.

25 (U) 83 10 - 84 09 Excellent progress was made elucidating species of the Dirus, Maculatus and Philippinensis species complexes of Anopheles by morphology, cytogenetics and cross mating studies. A revision of Culex (Culex) of the Middle East is nearly complete and several new species of Aedes (Stegomyia) from Africa were detected. Collections in Israel and Sierra Leone resulted in over 12,000 specimens for study. Over 5,300 collection forms and data on 85,793 specimens were incorporated into a computer data base. 9 papers were published, 9 formal presentations were made and 12 manuscripts are in press. For technical report see Walter Reed Army Institute of Research Annual Report 1 Oct 83 to 30 Sep 84.

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Project 3M162770A870 MEDICAL DEFENSE AGAINST INFECTIOUS DISEASE

Work Unit 042 Biosystematics of Arthropods of Military Medical Importance

Investigators:
Principal: Bruce A. Harrison, LTC, MSC
Assistant: Ralph E. Harbach, CPT, MSC; E.L. Peyton; Y.-M. Huang, Ph.D.; Thomas J. Zavortink, Ph.D.
Associate: SP4 Richard Soltero, Thomas V. Gaffigan, Charlotte Burnett, James E. Pecor, Dolores Chalfant, Olimpia Areizaga

Problem

Epidemiological studies and disease control strategies involving arthropod-borne pathogens are dependent upon biosystematic research support to provide accurate identifications of arthropod vectors and reservoirs. The objectives of biosystematic research of medically important arthropod groups are (1) to resolve systematic problems by identifying all of the species in these groups, (2) to describe and illustrate all the species in these groups, (3) to develop effective keys for identifying all of the species under study in their 4 primary life stages, (4) to analyze biological and ecological data useful in understanding the epidemiology of diseases and in the control of vector species, (5) to provide data concerning the medical importance of each species under study, and (6) to train personnel in field and laboratory techniques and in systematic research methods. All current research efforts are focused on mosquito vector groups that are involved significantly in the transmission of human pathogens: (1) malaria vector-groups of the Oriental Region Leucosphyrus and Maculatus Groups of Anopheles (Cellia), and of northern Africa and southwest Asia (genus Anopheles), (2) arbovirus and filaria vectors of northern Africa and southwest Asia Culex (Culex), and of the Ethiopian Region Aedes (Stegomyia), and (3) arbovirus vectors of the Neotropical Region (genus Trichoprosopon). An effort accompanying these studies, is the development of a computer based master file of detailed systematic and ecologic data for the one million plus mosquito specimens and their collection records in the National Museum of Natural History. This effort is directed at providing easily accessible and coordinated ecological, distributional and taxonomic data to military, public health and other scientific and environmental personnel or organizations for use in epidemiological or vector control schemes.

Progress

Research on the Leucosphyrus Group of *Anopheles* (*Cellia*) in the Oriental Region was highly productive this year. This study is a collaborative effort involving researchers at the AFRIMS Laboratory and at Mahidol University in Bangkok, Thailand. Until this year the collaborative cytogenetic and ecological studies in Thailand had been impeded seriously by highly variable morphological characters on the adults, which prevented field identification of specimens. After a lengthy examination and measurement of numerous potential characters on thousands of specimens of species either confirmed by cytogenetics or by immature characters, keys were prepared this year for the adult females which will identify accurately the 9 species of this group that occur in Thailand. Refined keys for the 4th larval instar and pupal stage also were prepared with the adult key and sent to the Thailand collaborators. These keys were very well received and have aided ongoing field efforts significantly. One group of characters found having significant differences in the adult females are the cibarial and maxillary teeth. Results show that there is a degree of overlap or similarity in the cibarial and maxillary teeth between some species or forms. There also appears to be a relatively narrow range of variation in the number of teeth for each species. Actually, the length and character of the cibarial teeth in some species appear distinct and may be more useful for species discrimination in some cases than the number of teeth present. These intensive morphological examinations have also enabled the investigator to evaluate relationships among the species within the group. Accordingly, a new classification of subgroups and species complexes has been formulated for the Leucosphyrus Group.

The overall picture of the Leucosphyrus Group in the Oriental Region nearly seems resolved, with 20 species now recognized where only 13 species, subspecies and forms were recognized just 7 years ago. Furthermore, the distinct species status of most of the new members originally recognized only by small morphological differences has now been confirmed by karyotype, polytene chromosome, electrophoresis and/or crossing studies by our collaborators in Thailand. In fact, at least 5 manuscripts are currently in press that describe some of these recently discovered differences. The exceptionally rapid elucidation of this highly complex group of sibling species stands in contrast to the amount of time taken for the famous Maculipennis and Gambiae Complexes to be resolved. Adults of these latter 2 complexes still cannot be identified by morphological characters after 60 plus and 20 plus years of work, respectively, by numerous researchers. The taxonomic resolution of the Leucosphyrus Group in such a short period of time definitely can be attributed to a well conceived plan with an integrated taxonomic approach utilizing morphological, crossing, genetic and biochemical techniques. This approach has allowed the taxonomist to define species parameters by the examination of progeny broods from mothers identified by genetic or biochemical techniques.

Although the species parameters in the Leucosphyrus Group are fairly well documented, considerable work still remains in describing the individual species and preparing other sections of the monograph. Meanwhile the efforts being expended by our collaborators in Southeast Asia to further define this complex and its impact on malaria transmission in Southeast Asia now includes funds from a minimum of 5 grants funded by the WHO, USAID, NAS(BOSTID) and the U.S. Army. One of these projects, a longitudinal malaria study in Chantaburi Province, Thailand, being conducted by AFRIMS personnel, is now trying to determine the actual involvement and impact of *dirus* A and *dirus* D on malaria transmission in an area with a high malaria endemicity. The impact the newly found sibling species in the Leucosphyrus Group could have on our knowledge of malaria epidemiology may be illustrated best by the situation in Thailand. Prior to our revisionary efforts on the Leucosphyrus Group one species, *balabacensis*, was considered the primary vector of malaria in Thailand. Now 5 species have been identified in Thailand from what was originally called *balabacensis*. These 5 species have distinct distributions, biological habitats and behavior patterns, and almost certainly are not equally important in the transmission of malaria.

Work on the Maculatus Complex of *Anopheles* (*Cellia*) in Thailand continued. Collaborators in Thailand were able to make a key for 3 of the "*maculatus*" sibling species that occur in that country, however, a search is continuing for even better characters. Three papers characterizing chromosome differences in the sibling species occurring in Thailand are currently in press. The impact of this sibling complex on malaria transmission in Thailand still needs to be determined.

Studies on the *Anopheles* of the Middle East were enhanced by the collection of an additional 295 adult specimens of 6 species in Israel. Most of these specimens have associated immature skins. Plans also were initiated to prepare pictorial keys for the anophelines of this region. Pencil illustrations were made for the larva and pupa of a new species of *Anopheles* from Egypt and also for a new species of *Anopheles* from Sri Lanka. A close examination of the immature stages of Egyptian Anopheles collected in 1983, led to the discovery of a 3rd new species of *Anopheles* which may be restricted to southern Africa.

On 1 August 1984, a Smithsonian post-doctoral fellow arrived at the Walter Reed Biosystematics Unit to begin work on developing a key that will identify the adult females of *Anopheles* (*Nys.*) *evansae*, *nuneztovari*, *rangeli* and *trinkae* on the eastern slopes of the Andes Mountains in South America. This special project was initiated in response to a number of requests for assistance in identifying these members of the Albimanus Section Complex from South America. Apparently, the currently published keys for these species have been found inadequate by malaria control personnel in several countries. This appears to be particularly true in the eastern slopes of the Andes of several countries. Peru and Columbia currently are experiencing major malaria epidemics in these regions, and the malaria control personnel have been unable to identify the species they are trying to control. Preliminary evidence indicates that the 4th larval instar is the best stage for identifying these species. Accordingly, adults reared with associated immature skins are being studied and attempts are being made to obtain additional reared specimens with skins.

Work on the revision of the genus *Trichoprosopon* from Central and South America continued until the responsible taxonomist resigned his position with the WRBU on 30 June 1984. All the plates for this study except one have been completed. Work remaining to be done involves writing descriptions and other sections of the monograph.

Research on a revision of the *Culex* (*Culex*) from Southwest Asia and Egypt is nearing completion. This assemblage of 20 species involves at least 10 species that are involved or implicated in the transmission of pathogens that cause filariasis and arboviral diseases (e.g., *pipiens*, *quinquefasciatus*, *perexiguus*, *theileri*, and *tritaeniorhynchus*). Progress includes the completion of: 37 plates of illustrations, 20 adult descriptions, a key to the adults (males and females), and 12 larval and 8 pupal chaetotaxy tables. Of particular interest to culicidologists will be a new layout and representation of the various parts of the male genitalia. These illustrations will more accurately represent the various structures of taxonomic significance than previously published illustrations. Portions nearly completed are: 4 additional pupal chaetotaxy tables, pictorial keys, generic keys, subgeneric keys for the genus *Culex*, and the synonymy section for each species. Work is continuing on the immature descriptions and other sections of the revision, and a completed first draft is anticipated in FY85.

During the year the taxonomist working on the *Culex* (*Culex*) prepared 2 manuscripts of great importance to systematists working on the Pipiens Complex. The first was recently published (July) and resolves the long standing problem of how to deal with the physiological variants of pipiens that are autogenous and bite man, and usually are ascribed the name, molestus Forskal. This paper completely reviews the taxonomic status of molestus in light of morphological, behavioral/physiological and crossing variations. A neotype was designated and described for molestus, and the use of the name, molestus, for behavioral/physiological variants of pipiens was considered unjustified. The second paper, designating and describing a neotype for pipiens, is currently in press. The publication of these 2 papers will permanently fix the identities and synonymy of pipiens and molestus and lend considerable stability to the nomenclature of the Pipiens Complex.

Revisionary efforts on the African Aedes (Stegomyia) mosquitoes during FY84 were concentrated on collections from West Africa, and on resolving species problems in the Africanus and Simpsoni Subgroups. Several members of these subgroups are important in the zoonotic and human cycles of Dengue, Chikungunya and Yellow Fever viruses in Tropical Africa. A very successful 75 day collecting trip to Senegal and Sierra Leone was accomplished. This trip resulted in nearly 500 specimens from Senegal and nearly 1800 adults reared with associated immature skins from Sierra Leone. The specimens from Senegal were borrowed from Dr. M. Cornet, ORSTOM, during a brief visit to Senegal before collecting in Sierra Leone. The specimens from Lierra Leone came from 208 collections made in 4 localities in 2 provinces, and represent at least 40 species in 7 genera. Two new species (1 Aedes and 1 Culex) and 6 new species records for Sierra Leone were included in the specimens. Of even greater significance, topotypic specimens were collected for 3 Stegomyia species, Aedes aegypti formosus, Ae. africanus and Ae. blacklocki. These specimens are extremely valuable to this revisionary study.

Two important manuscripts on Aedes (Stegomyia) mosquitoes were prepared during the year. One, a new species in the Africanus Complex, will be submitted for publication in early FY85. The second is a very significant paper, as it correctly identifies Aedes (Stg.) bromeliae as the Yellow Fever vector in East Africa, instead of Aedes simpsoni. This paper is being submitted to the Journal of Medical Entomology in early FY85.

At the end of June one of the professional entomologists resigned his position, and subsequently that authorization was lost. That taxonomist had been studying the Aedes subgenus Neomelaniconion in support of USAMRDC research in Africa on Rift Valley Fever virus. Since the departure of that professional, the taxonomist working on the *Aedes* (*Stegomyia*) has begun an examination and comparison of African and Philippine material of *Aedes* (*Neo.*) *lineatopennis* to determine if they are conspecific. The type of this species is from the Philippines.

A total of 220 man-days involving 2 professionals and 2 technicians were spent on 3 major field trips during the year: Senegal 45 days, Israel 30 days and Sierra Leone 73 days. Nearly 5100 adults, with approximately 10,000 associated immature skins and whole larvae were collected. These specimens are extremely valuable to the ongoing *Aedes*, *Anopheles*, and *Culex* projects, and in some cases were the first specimens of critically needed species. During these trips, 6-7 local national technicians/graduate students were trained in field collecting and taxonomic techniques. In addition, the 2 WRBU technicians received very valuable field experience and contributed very significantly to the success of each trip.

During the year 14,560 specimens were received by the unit as gifts, transfers, loans, etc., for deposition in the National Museum of Natural History, or for use in ongoing studies and then return to the loaning institution. Outgoing loans, exchanges or gifts involved 9240 specimens. Approximately 17,979 specimens were identified by unit personnel for DOD units, public health organizations and the U.S.D.A. from the following countries: Argentina, Cameroon, Costa Rica, Egypt, Guyana, Honduras, Israel, Kenya, Peru, Senegal, Sierra Leone, Somalia, Sri Lanka, Sudan, Thailand, Trinidad, Tunisia, Turkey, Uganda and the U.S.A.

The computer data base was increased by 5302 collection forms and 85,793 specimens during the year. The South American File was completed except for Brazil. Important entries were made for Egypt and Israel in the Middle East file. Service demands for information and maps declined sharply to slightly over 40 requests from the 150 made last year. This decline is due to a re-emphasis on data entry with a concurrent de-emphasis on responding to requests. These changes are in response to recommendations made by an external review team that visited the project in February (see Annual Report dated August 1984 for Mosquito Information Management Project). One MIMP Technician resigned from her position as of 8 June. Recruitment for a replacement is underway.

Personnel in the unit were involved in a number of other aspects of biosystematic and medical entomology research. (1) Approximately 28 international visitors from 14 countries visited the facilities and were assisted in many ways, to include training. (2) One professional organized a symposium on "Mosquito Systematics" at the annual meeting of the American Mosquito Control

Association in Toronto, Canada. (3) One professional translated a publication from Chinese to English, and also served as a special Smithsonian host for a visiting Chinese delegation. (4) One member served on an USAID scientific peer review panel for proposals submitted to the Program of Science and Technology Cooperation (PSTC). (5) One member served as a special consultant on taxonomy for a meeting in Panama sponsored by the National Research Council, National Academy of Science. (6) Two members served as guest lecturers for a week long course at the Wedge (Univ. of S. Carolina) in Charleston, S.C. (7) The professional staff reviewed 35 manuscripts being submitted for publication in internationally recognized periods, and 6 research proposals being submitted to various government funding agencies. (8) The unit sent keys, publications, instructions and/or collecting and rearing equipment to 4 Army entomologists working in Panama and Honduras, 3 Navy entomologists in Egypt and the Philippines and provided numerous supplies for USAMRIID trips into Senegal and the Central African Republic.

Future plans

Research currently in progress on the Leucosphyrus Complex of *Anopheles*, the *Aedes* (*Asegomyia*) of the Afrotropical region, the *Anopheles* (*Nyssorhynchus* of the eastern slopes of the Andes, the *Anopheles* of the Middle East and the *Aedes* (*Neomelaniconion*) species problems in Africa will continue. The project on the *Culex* (*Culex*) of Southwest Asia and Egypt will be terminated during FY85 when the investigating officer is reassigned. A new officer to replace this departing professional will arrive in January 85. The project for this new officer remains to be determined. FY85 should also see a resolution and completion of the post-doctoral study of the *Anopheles* (*Nyssorhynchus*) of the eastern slopes of the Andes. Descriptions for at least 4 new species are anticipated for publication this next year. Provided sufficient material is available, a resolution of the status of *Aedes* (*Neo.*) *lineatopennis* in Kenya is anticipated. At least 2 collecting trips are anticipated, in Peru and the Ivory Coast. Ongoing collaborative efforts with Egypt, Israel, the WRAIR overseas laboratories, USAMRIID and the Navy overseas laboratories will continue.

Publications

Mendis, K. N., R. L. Ihalamulla, E. L. Peyton, and S. Nanayakkara. 1983 (1984). Biology and descriptions of the larva and pupa of *Anopheles (Cellia) elegans* James (1903). Mosq. Syst. 15(4):318-324. (March)

Reinert, J. F., and S. Ramalingam. 1983 (1984). *Aedes (stegomyia) platylepidus*, first description of the pupa and larva (Diptera:Culicidae). Mosq. Syst. 15(4):337-344. (March)

Zavortink, T.J. 1984. Book Review: Frank, J.H. and L.P. Lounibos (ed.) 1983. Phytotelmata. Terrestrial plants as hosts for aquatic insect communities. Mosq. News 44(1):129. (March)

Harbach, R. E., B. A. Harrison and A. M. Gad. 1984. *Culex (Culex) molestus* Forskol (Diptera: Culicidae): neotype designation, description, variation, and taxonomic status. Proc. Entomol. Soc. Wash. 86(3):521-542. (July)

Reinert, J. F. 1984. Medical Entomology Studies - XVI. A review of the species of subgenus *Verrallina*, genus *Aedes*, from Sri Lanka and a revised description of the subgenus (Diptera: Culicidae). Mosq. Syst. 16(1); 1-130. (July)

Peyton, E. L., R. E. Harbach and D. R. Roberts. 1984. Culex *(Melanoconion) serratimarge* (Diptera: Culicidae), a new occurrence record from Bolivia. Mosq. Syst. 16(2):183-184. (September)

Harbach, R. E., E. L. Peyton and B. A. Harrison. 1984. A new species of *Culex (Melanoconion)* from southern South America (Diptera: Culicidae). Mosq. Syst. 16(2):185-200. (September)

Harrison, B. A. and E. L. Peyton. 1984. The value of the pupal stage to anopheline taxonomy, with notes on anomalous setae (Diptera: Culicidae). Mosq. Syst. 16(2):201-210. (September)

Ward, R. A. 1984. Chapter 8. Mosquito fauna of Guam: Case history of an introduced fauna. pp. 143-162 in Laird, M. (ed.). Commerce and the spread of pests and disease vectors. Praeger Scientific, N.Y. 354 p.

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Zavortink, T.J. 1984. Ecology of North American treehole mosquitoes. Mosquito Ecology Workshop, 8-12 Jan., Orlando, FL.

Harrison, B.A. 1984. Army vector biosystematic studies and support. Biennial Army Med. Entomol. Conf., 6 Mar., San Antonio, TX.

Harrison, B.A. 1984. Mosquito taxonomy and identification. Course taught at Wedge, Univ. of S. Carolina, 12-14 Mar., Charleston, S.C.

Peyton, E.L. 1984. Mosquito biology, ecology and preparation techniques. Course taught at Wedge, Univ. of S. Carolina, 15-16 Mar., Charleston, SC.

Harbach, R.E. (with B.A. Harrison, A.M. Gad, M.A. Kenawi and S. El Said). 1984. Report on recent mosquito taxonomic studies in Egypt. Annual meeting of Am. Mosq. Contr. Asoc., 20 Mar., Toronto, Canada.

Harrison, B.A. (with R.E. Harbach). 1984. *Culex* (*Thaiomyia*) Bram 1966, a synonym of *Culex* (*Culiciomyia*) Theobald 1907, with observations on morphological variability found in *Culiciomyia*. Annual meeting of Am. Mosq. Contr. Assoc., 20 Mar., Toronto, Canada.

Zavortink, T.J. 1984. Speculations on Sabethine Taxonomy. Annual meeting of Am. Mosq. Contr. Assoc., 20 Mar., Toronto, Canada.

Harrison, B.A. 1984. Observations of a mosquito collector in Egypt. Monthly meeting of Entomol. Soc. Washington, 5 Apr., Washington, DC.

Peyton, E.L. 1984. Taxonomic problems. BOSTID Research Grants Program Coordination Meeting, 27 Aug., Panama City, Panama.

Manuscripts in Press (TOTAL 13)

1. Baimai, V., R.G. Andre and B.A. Harrison. 1984. Heterochromatin variation in the chromosomes in Thailand populations of Anopheles dirus A (Diptera: Culicidae). Can. J. Genet. Cytol. (in press)

2. Baimai, V., C.A. Green, R.G. Andre, B.A. Harrison and E.L. Peyton. 1984. Cytogenetic studies of some species complexes of Anopheles in Thailand and Southeast Asia. Southeast Asian J. Trop. Med. Pub. Hlth. (in press)

3. Faran, M.E., C. Burnett, J.J. Crockett and W.L. Lawson. 1984. Computerized information and collection management system for systematic research and medical entomology (Diptera: Culicidae). Mosq. Syst. 16: (in press)

4. Green, C.A., V. Baimai, B.A. Harrison and R.G. Andre. 1984. Cytogenetic evidence for a complex of species within the taxon, Anopheles maculatus (Diptera: Culicidae). Biol. J. Linn. Soc. (in press)

5. Green, C.A., B.A. Harrison, T.A. Klein and V. Baimai. 1984. Cladistic analysis of polytene chromosome rearrangements in anopheline mosquitoes, subgenus Cellia, Series Neocellia. Syst. Zool. (in press)

6. Harbach, R.E., C. Dahl and G.B. White. 1985. Culex (Culex) pipiens Linnaeus (Diptera: Culicidae): concepts, type designations, and description. Proc. Entomol. Soc. Wash. 87: (in press)

7. Klein, T.A., B.A. Harrison, V. Baimai and V. Phunkitchar. 1984. Hybridization evidence supporting separate species status for Anopheles nivipes and Anopheles philippinensis. Mosq. News 44: (in press).

8. Klein, T.A., B.A. Harrison, J.S. Grove, S. Vongradist and R.G. Andre. 1985. Correlation of survival rates of Anopheles dirus A (Diptera: Culicidae) with different infection densities of Plasmodium cynomolgi. Bull. W.H.O. (in press)

9. Linthicum, K.J. 1984. Mosquito Studies (Diptera, Culicidae) - XXXVII. A revision of the Argyritarsis Section of the subgenus Nyssorhynchus of Anopheles. Contr. Am. Entomol. Inst. (Ann Arbor). 21: (in press)

10. Roberts, D.R., E.L. Peyton, F.P. Pinheiro, R. Vargas and F. Balderama. 1985. The associations of arbovirus vectors with gallery forests and domestic environments in southeastern Bolivia. Bol. Sanit. Panam. (in press)

11. Ward, R.A. 1984. Second supplement to "A Catalog of the Mosquitoes of the World" (Diptera: Culicidae). Mosq. Syst. 16: (in press)

12. Watts, D.M., B.A. Harrison, S. Pantuwatana, T.A. Klein and D.S. Burke. 1985. Failure to detect natural transovarial transmission of dengue viruses in Aedes aegypti and Aedes albopictus (Diptera: Culicidae). J. Med. Entomol. (in press)

13. Harbach, R.E. 1984. Book Review: Zaman, V. 1983. Scanning electron microscopy of medically important arthropods. Mosq. News 44(3): (in press)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OA 6442
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISS'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A870	AE	044 WWGD
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code): (U) Rickettsial Diseases of Military Personnel

12. SUBJECT AREAS: 0613 Microbiology 0603 Biology

13. START DATE: 55 08
14. ESTIMATED COMPLETION DATE: Cont
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-house

17. CONTRACT/GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT/GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	5.0	445
85	3.0	632

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code): Washington, D C 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL: TOP, F H JR
d. TELEPHONE NUMBER (include area code): (202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME: Division of CD&I
b. ADDRESS:
Walter Reed Army Institute of Research
Washington, D C 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR: HEDLUND, K W
d. TELEPHONE NUMBER (include area code): (202)-576-2146
f. NAME OF ASSOCIATE INVESTIGATOR (if available): Jerrells, T R MacMillan, J G
g. NAME OF ASSOCIATE INVESTIGATOR (if available): Rice, R M Mentes, W R Jarboe, D L

21. GENERAL USE: FINA
MILITARY.CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Rickettsial Infections; (U) Laboratory Diagnosis; (U) Vaccines; (U) Epidemiology; (U) Lab Animals (U) Mice; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Develop experimental rickettsial immunogens; define the pathology of rickettsial infections in laboratory animals to include subhuman primates; determine the sequence of events leading to immunity following vaccination or infection. These studies are aimed at development of vaccines that will protect deployed military troops, and development of immunoassays to evaluate the extent of immunity induced by vaccination.

24. (U) The propagation of rickettsiae in various new cell lines in tissue culture to optimize rickettsial production. Development of rapid and more sensitive assays of intact rickettsiae and the individual immunogenic components of rickettsiae for vaccine use. Development of T cell hybridomas as correlates of cell mediated immunity. Determination components which elicit protective responses. Characterization of cell mediated and humoral responses to individual immunogens in development of protective immunity.

25. (U) 83 10 - 84 09 An immunological memory detectable by gamma interferon production was demonstrated in previous scrub typhus infected primates which lasts for up to one year, but not as long as five. These same primates on rechallenge form eschars but do not develop rickettsemia. Mice immunized by subcutaneous infection with scrub typhus and subsequently rechallenged with irradiated whole organisms 28 days later showed circulating interferon gamma which peaked 4 hrs after inoculation. Using thymus bearing mice, it was found that passive transfer of delayed-type hypersensitivity could be accomplished Lyt-1 (Helper) T cells establishing a readily measurable parameter of cell mediated immunity and the immune mechanisms of reinfection. T cell clones and hybridoma which react specifically with rickettsial antigens are being generated both as immunologic probes and as a source of potentially useful by-products. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project 3M162770A870 MEDICAL DEFENSE AGAINST INFECTIOUS DISEASE

Work Unit 044 Rickettsial Diseases of Military Personnel

Investigators:

Principals:
COL Kenneth W. Hedlund, MD
Thomas R. Jerrells, PhD
MAJ Robert M. Rice, VC
CPT James G. MacMillan, VC
CPT Daniel L. Jarboe, VC

Associates:
SP5 September Bodden
SP4 Mirian R. Pedersen
SP4 Stephan E. Platt
SP4 Peter F. Pond

Problems and Objectives:

Immunity to rickettsiae is enhanced by both "T" cell lymphocytes and humoral antibodies; however, the principal effector of rickettsial killing is the macrophage. Lymphokines may play an important role. Undoubtedly there is a complex in vivo interaction between the immune, phagocytic and inflammatory host defenses against these obligate intracellular bacteria. Future progress in research on rickettsial immunity and protection from disease will depend upon the application of modern methods of cell biology to identify the rickettsial antigens, their mode of presentation to the host and the essential components of the immune response which must be stimulated to afford long lasting protection against rickettsial infection. The objectives of this work unit is to characterize the key components of the immune response and to identify the rickettsial antigens which trigger these critical immunoprotective responses.

Progress:

The basic mechanisms of the pathology of and immunity to *Rickettsia tsutsugamushi* have been greatly clarified by the finding that inbred strains of mice are susceptible to lethal infection with this rickettsia. It was shown that protection against lethal infection could be passively transferred to naive syngeneic mice by using spleen lymphocytes or, further, by a cell population enriched for thymus-derived lymphocytes (T-cells) obtained from spleens or peritoneal exudates. These studies have been used to argue the central role of cell-mediated immunity in acquired immunological resistance to reinfection with *R. tsutsugamushi*. In more recent studies athymic (nu/nu) and thymus-bearing mice have been used to investigate the nature of the cell type mediating passive transfer of resistance to infection with *R. tsutsugamushi*. Transfer of immune spleen cells obtained from thymus-bearing mice reconstituted the ability of athymic animals to resist intraperitoneal challenge. That this protection was mediated by T-cells was suggested by data demonstrating protection with a T-cell-enriched population and, further, by the removal of protective capacity by treatment with anti-Thy 1.2 serum and complement. Since T-cells have been functionally separated into distinct subpopulations based on Lyt surface antigens, it was of interest to determine the T-cell subpopulation capable of transferring resistance. Depletion of the T-helper-amplifier cell population with a monoclonal anti-Lyt 1.2 antibody abolished the ability to transfer immunity to athymic mice, but treatment with anti-Lyt 2.2 had no effect. By using thymus-bearing mice it was found that passive transfer of delayed-type hypersensitivity, a well-established parameter of cell-mediated immunity, could be accomplished with T-cells and with Lyt 1-bearing T-cells, establishing a link between readily measurable parameters of cell-mediated immunity and the immune mechanisms mediating protection against reinfection.

Further studies have employed the ability of T-cells to proliferate in response to specific antigens. This assay was used to probe the T-cell response to heterologous antigens in an attempt to define the T-cell clones arising after immunization. Data clearly demonstrated that the heterologous cross-protection that has been noted in mice immunized with a single strain of *R. tsutsugamushi* is reflected in the cross-reactivity at the T-cell level. One interesting observation resulting from this study was the fact that maximal lymphocyte proliferation in response to antigen was not correlated with the development of protective immunity. Since lymphocyte proliferation developed relatively late, it was suspected that active suppression of lymphocyte responses occurs during the development of a chronic immunizing infection. To test this hypothesis, the responses of spleen cells obtained from infected and noninfected mice to the T-cell mitogen concanavalin A were examined.

It is interesting that spleen cells treated to remove adherent cells demonstrated an enhanced response to concanavalin A which was nearly the same as that of cells from noninfected mice. The addition of indomethacin, an inhibitor of prostaglandin synthesis, restored the proliferative response to both mitogens and antigens. The proliferative response to Karp antigen required the presence of macrophages presumably acting as antigen-presenting cells, which has been shown for T-dependent antigens. By using a cell population enriched for macrophages it was also possible to suppress the responses of noninfected spleen cells to concanavalin A and lipopolysaccharide. In further studies it was found that this cell population contained a population of highly activated macrophages as measured by tumor cell cytolysis or cytostasis suggesting that the suppression of lymphocyte proliferation may be the result of the development of a population of activated macrophages.

In recent studies, macrophage membrance components including Ia antigens (Steeg, et al.) and receptors for the Fc fragment of immunoglobulins (Vogel, et al.) have been shown to be modulated by gamma interferon (IFN-γ). Furthermore, IFN-γ has been also shown to activate macro-phages in terms of cytolysis and cytostasis.

It is relatively easy to propose a model system for the role of IFN-γ in the responses of immune mice or during the development of primary immunity. Antigen reactive T-cells produce IFN-γ (as well as many other lymphokines) which (i) acts on the inflammatory macrophages to induce synthesis of Ia antigen, which serves to amplify the response by antigen presentation to other antigen-specific T-cells, and (ii) activates the inflammatory macrophage to become rickettsiacidal as well as possibly interacting with other cell types to inhibit rickettsial growth, as described by Turco and Winkler. It is also possible that IFN-γ may act as an immunoregulatory molecule, either indirectly, by activating macrophages which produce prostaglandins, or directly, by acting on proliferating lymphocytes as has been shown. The immunoregulatory role of IFN-γ in this system is currently under study in this laboratory, and no direct evidence exists for this arm of the model.

In summary it is clear that T-cells with the Lyt 1 surface phenotype play a pivotal role in protective immunity as well as in the mediation of delayed-type hypersensitivity to R. tsutsugamushi in a murine model. The fact that the animals serving as immune cell donors are chronically infected and thus are constantly being stimulated by antigen may have an influence on the nature of the T-cell type mediating immunity should help answer this question. An important aspect of immunity to R. tsutsugamushi, which apparently occurs after interaction of immune T-cells and antigen, is the production of lymphokines, including IFN-γ, which interact with macrophages to amplify the immune response by inducing Ia-antigen-bearing, antigen-presenting cells and to activate macrophages to become rickettsiacidal. It is felt that the interaction of macrophages with lymphokines results in a population of macrophages which serve to negatively regulate the immune response. Further studies of these systems will answer important questions

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concerning the role of the immune system in the development of chronic rickettsial infections.

Recommendations:

We have established the rather sophisticated assays for the production of the lymphokine IFN-γ *in vitro* and *in vivo* following a whole organism challenge. We need to perservere in our efforts to optimize the production of rickettsiae in our tissue culture systems so as to provide a fundamental base for the definition of which rickettsial epitopes are eliciting the key protective responses.

This particular work unit has two components, the first involves the production and characterization of rickettsiae propagated either in embyronated yolk sacs or in tissue culture. Once these rickettsiae are harvested and quantiified they are then used as intact whole organisms either as an infectious agent or as an attenuated immunogen after irradiation. So far the sophisticated cell mediated and lymphokine studies outlined in the progress section have reflected the host response to the intact organism. We are progressing to the next stage in which a clear definition of which rickettsial antigens elicit a protective response will be made. To do this we are optimizing our tissue culture methodology to maximize consistent production yields. It has recently become apparent that solubilization of intact rickettsiae followed by isoelectrofocusing techniques can provide us with a large variety of partial purified antigenic rickettsial polypeptides which will be studied for their protective ability. Correlations between *in vivo* survival and *in vitro* cell mediated phenomena can in the future be more clearly ascribed to particular rickettsial antigen or group of antigens which will be a important step in defining the nature of a protective vaccine. In addition, in as much as T cell responses are generally now recognized to play pivotal role in the immunologically keyed survival of the challenged hosts, we are developing "T" cell clones and hybridomas both as diagnostic probes and a potential source of cellular products of particular protective importance.

Formal Presentations:

1. Palmer, B.A., Hetrick, F.M., and Jerrells, T.R.: *In vitro* production of IFN-γ by splenic T-lymphocytes correlates with protective immunity to *Rickettsia tsutsugamushi*. 4th Annual Meeting of the American Society for Rickettsiology and Rickettsial Diseases, Airlie, VA, October 1983.

2. MacMillan, J.G., Palmer, B.A. and Jerrells, T.R.: Immunological responses of cynomolgus monkeys (*Macaca fasicularis*) to intradermal and subcutaneous infection with the Karp strain of *R. tsutsugamushi*. 4th National Meeting of the American Society of Rickettsiology. Airlie, VA, October 1983.

3. Jerrells, T.R. and Palmer, B.A.: Immunosuppression associated with the development of chronic *R. tsutsugamushi* infections of BALB/c mice. 4th National Meeting of the American Society of Rickettsiology. Airlie, VA, October 1983.

4. Rice, R.M., Jerrells, T.R., Bernier, R.D. and Diggs, C.L.: Ability of an irradiated tissue culture grown *Rickettsia tsutsugamushi* vaccine to induce humoral and cell mediated immunity. American Society of Tropical Medicine and Hygiene. San Antonio, TX, December 1983.

5. Jerrells, T.R. and Palmer, B.A.: Regulation of Ia bearing macrophage inflammatory responses during lethal infection with *Rickettsia tsutsugamushi*. 16th International Leuckocyte Culture Conference. Cambridge England, August 1984.

6. Jerrells, T.R.: Inhibition of Ia antigen expression on inflammatory macrophages in lethal *Rickettsia tsutsugamushi* infections. FASEB Summer Conference, Antibody and lymphocyte networks: Impact of infectious agents. Saxon River, VT, August 1984.

7. Jerrells, Thomas R. and William H. Bancroft: Immunosuppression associated with the development of chronic infection of BALB/c mice with *Rickettsia tsutsugamushi*. Symposium, Clinical Tropical Medicine, ACAV open meeting, San Antonio, TX.

Publications:

1. Jerrells, T.R. Association of an inflammatory I region-associated antigen-positive macrophage influx and genetic resistance of inbred mice to *Rickettsia tsutsugamushi*. Infect. and Immun. 42:549-557, 1983.

2. Jerrells, T.R. and Osterman, J.V. Parameters of cellular immunity in acute and chronic *Rickettsia tsutsugamushi* infections of inbred mice. *In* host defenses to intracellular pathogens. Eisenstein and Actor (eds) Plenum Press, New York, pp. 355-360, 1983.

3. Palmer, B.A., Hetrick, F.M. and Jerrells, T.R. Production of immune (γ) interferon in mice immune to *Rickettsia tsutsugamushi*. Infect. and Immun. 43:59-65.

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1. Steeg, P.S., R.N. Moore, H.M. Johnson, and J.J. Oppenheim. 1982. Regulation of murine macrophage Ia antigen expression by a lymphokine with immune interferon activity. J. Exp. Med. 156:1780-1793.

2. Vogel, S.N., L.L. Weedon, R.N. Moore, and D.L. Rosenstreich. 1982. Correction of defective macrophage differentiation in C3H/HeJ mice by an interferon-like molecule. J. Immunol. 128:380-386.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION DA OB 6535	2. DATE OF SUMMARY 841001	REPORT CONTROL SYMBOL DD-DR&E(AR) 636				
3. DATE PREV SUM'RY 831001	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISS'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A870	AJ	045 WWM5
b. XXXXXXXXXXX				
c. XXXXXXXXXXX	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Exploratory Development of Anti-Parasitic Disease Drugs

12. SUBJECT AREAS
0615 Pharmacology 0603 Biology 0703 Organic Chemistry

13. START DATE 6607	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	9.0	1,074
85	9.5	950

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H Jr
d. TELEPHONE NUMBER (include area code)
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
Division of Experimental Therapeutics
b. ADDRESS
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
DAVIDSON, D E
d. TELEPHONE NUMBER (include area code)
(301)-427-5411
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
REID, W A
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE
FINA
MILITARY CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U)Malaria; (U)Leishmaniasis; (U)Trypanosomiasis; (U)Schistosomiasis; (U)Drug Development; (U)Chemistry; (U)Pharmacology; (U)Biology; (U)Lab Animals; (U)RAD I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23.(U)Design, test & develop new drugs with chemotherapeutic or chemoprophylactic activity against parasitic diseases which pose a threat to DOD personnel and/or the accomplishment of military operations. Develop laboratory model test systems.

24.(U)Chemical samples obtained from a rational synthesis program, industry & academia are tested for activity against diseases. Information is used in buiding new synthesis and selecting compounds for clinical trials. New test systems are developed. Candidates under preliminary pharmacological testing.

25.(U)8310-8409 Approximately 2000 new samples were obtained for testing. In vitro antileishmanial activity of purine analogues & WR6026 metabolites were determined. Formulation and in vivo and in vitro testing of encapsulated formycin & pentamidine as antileishmanial agents were initiated. An in vivo screening system for topically applied antileishmanial compounds was established & some emphasis was shifted to this area of testing from testing against visceral leishmaniasis. WR6026 was evaluated as an effective antileishmanial agent in primates. Work continued on evaluating an RBC drug delivery system. Radiorespirometric methods were successfully applied to characterizing 23 new leishmania isolates, determination of drug susceptibility to chemotherapeutic agents and detection of G6PD in patients to receive antimalarial therapy. Rodent, primate and in vitro mode of action studies were completed on a new class of schistosome antipenetrants; a new use patent disclosure application was prepared for submission. The in vitro antrypanosomiasis drug screen was successfully re-established & 130 new compounds were tested. Data base systems for analysis, retrieval & reporting of biological & chemical test data were further developed. Metabolism & pharmacokinetic studies on absorption, distribution & excretion of WR6026, WR238,605 & artesunate were completed or are in progress. Methods for the in vitro quantitative assessment of interactions & activities of antimalarial drug combinations were developed. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83 - 30 Sep 84.

PROJECT: 3M162770A870 MEDICAL DEFENSE AGAINST INFECTIOUS DISEASE

WORK UNIT: 045 EXPLORATORY DEVELOPMENT OF ANTI-PARASITIC DISEASE DRUGS

INVESTIGATORS:

Principal: COL David E. Davidson, Jr., VC
Associates: LTC Willis A. Reid, Jr., MS
LTC Jonathan D. Berman, MC
MAJ Wilbur K. Milhous, MS
MAJ James K. Lovelace, MS
CPT Robert E. Miller, MS
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Bing T. Poon, Ph.D.
Ho Chung, Ph.D.

PROBLEM AND OBJECTIVES:

In many parts of the world where U.S. military personnel may be deployed, diseases such as malaria, leishmaniasis, schistosomiasis and trypanosomiasis are endemic. Prevalence of both falciparum and vivax malarias is increasing because of failing control and eradication efforts in many countries. In many of these areas, falciparum malaria has become resistant to currently available drugs, especially chloroquine. Current chemotherapy of leishmaniasis and trypanosomiasis is inadequate. There are no drugs available for prophylaxis, and those that are available for therapy have limited efficacy and dangerous side-effects. The objective of this work unit is the discovery and development of new drugs for prophylaxis and treatment of these diseases in military personnel. In-house research is complemented by and coordinated with contractor laboratory drug testing and research.

PROGRESS:

1. The Chemical Synthesis Program

Active contractual synthesis programs contributed to the anti-parasitic disease drug development efforts in the following proportions:

Malaria	3-contracts
Trypanosomiasis	2-contracts
Leishmaniasis	1-contract

This effort has also been supported by approximately 40% of the effort of three (3) preparation laboratories and a radiosynthesis laboratory. The balance of support from the preparation and radiolabeling laboratories is devoted to other drug development programs.

The 8-aminoquinolines continue to show encouraging blood and tissue schizonticidal activity against vivax malaria models. Work to investigate the value of substitution in various positions on the quinoline ring continues. Activity realized in these types is highly significant, and efforts to reduce their toxic effects have had some success. Contractors are attempting the total synthesis of qinghaosu and synthesis of analogs has been initiated. Quantities of it are being extracted from plants through in-house and contractual efforts, in order to provide for a comprehensive evaluation of this natural anti-malarial drug. In the search for alternative sources of artemisinin (qinghaosu), over 15 Artemisia species were examined without success for the presence of this material. A new sensitive detection method has been developed which permits rapid assay of the artemisinin content of Artemisia annua specimens.

While the 8-aminoquinoline WR 6026 remains the most promising anti-leishmanial drug, some nucleosides (Formycin B analogs) show encouraging signs of activity. Bis-amidines and imidazolium pyridines continue to perform best in the anti-trypanosomal screens.

Sixty-five newly synthesized compounds were submitted for biological testing, most of which had not been described previously in the chemical literature.

A reported mammalian metabolite of primaquine, 4-[8-[6-methoxyquinolinyl)-amino]pentanoic acid, can be detected in plasma of rats given pamaquine and can now be distinguished from pamaquine, desethyl-pamaquine, primaquine, and 8-amino-6-methoxyquinoline by high performance liquid chromatography. Scale-up of the isolation procedure to yield material for biological evaluation is in progress. Through use of HPLC, qinghaosu can now be separated from the mixed alcohols (early TLC eluters) or from the waxes (late eluters in TLC).

2. Data Processing, Data Base Development and Acquisition of Compounds

Work continued on the development of a user access system for an extensive data base of biology test input. A remote entry system was implemented for direct input of information from contractor laboratories. Biology edit, update and retrieval systems for schistosomiasis and malaria test systems were made operational; leishmaniasis data bases are estimated for completion by the end of FY 1985. The backlog of over 40,000 records of in vivo malaria test data was entered and updated in the data base, while the completion of input of over 23,000 more records is estimated for completion in 1985.

Approximately 2300 new chemical samples were acquired for antiparasitic disease drug testing. Over 9000 samples were shipped to screening systems for testing.

Work continues on the preparation of comprehensive compendia of malaria and schistosomiasis drug testing results.

3. Biological Testing/Studies

(**Malaria**) Investigations continued on the Chinese antimalarial qinghaosu derived from Artemisia annua. In vitro susceptibility testing suggested rapid inhibition of both protein and nucleic acid synthesis with well-defined concentration response curves generated after only 6-8h of drug exposure. Activity against isolates or clones of P. falciparum from patients with different clinical outcomes (including RI mefloquine and RII chloroquine and pyrimethamine failures) suggested an absence of cross-resistance with these classes of drugs. The interaction of qinghaosu in combination with quinine, mefloquine, chloroquine, pyrimethamine or halofantrine revealed a marked antagonism with chloroquine against all clones or isolates tested. Synergism with mefloquine was isolate dependent and only observed with mefloquine resistant isolates.

Procedures for the assessment of tetracycline activity were developed using a dual isotope (^{3}H-hypoxanthine and ^{14}C-isoleucine) method. By extending the period of drug exposure (from 24 to 48h prior to the addition of radioisotope) well-defined concentration curves could be generated. The interaction of quinine-tetracycline combinations with isolates of varying levels of susceptibility to either drug alone was judged to be independent or additive with regard to intrinsic activity. These procedures have been implemented by AFRIMS to investigate the roles of quinine and/or tetracycline susceptibility in treatment failures in Southeast Asia.

Methods to investigate the intrinsic activity of various plant extracts against P. falciparum in vitro were developed to guide in the extraction and identification of various natural products.

The two categories of antifol antimalarials, analogs of PABA and inhibitors of dihydrofolate reductase, were found to be antagonized by PABA and folic acid when using ^{3}H-hypoxanthine or ^{14}C-orotic acid as indices of parasite nucleic acid synthesis. These findings, combined with the observations that some parasite isolates could grow normally in medium without the addition of PABA and folic acid supplemented with dialyzed human plasma, suggested that earlier assumptions regarding the folate metabolism of P. falciparum are incorrect. Levels of folic acid 10-100 times less than physiological were found to markedly antagonize drug activity. These results suggest that resistance to antifol antimalarial drugs may involve multiple factors related to both the de novo synthesis of active folate cofactors and the ability to utilize exogenous erythrocyte folates in various forms.

Procedures were established for obtaining cloned P. falciparum parasites via direct visualization and micromanipulation.

(**Leishmania**) Radiorespirometry has continued to provide a sensitive method for the in vitro biochemical characterization of qualitative and quantitative metabolic differences of Leishmania species and strains. Twenty-three leishmanial isolates were tested bringing to 65 the total number characterized using this procedure. Radiorespirometry results compared favorably with the sensitivity obtainable using nucleic acid characterization; both procedures distinguished isolates below species level. Radiorespirometry has also been successfully applied to: a) determination of drug susceptibility of Leishmania to chemotherapeutic agents, b) detection of glucose-6-phosphate dehydrogenase in patients to receive 8-aminoquinoline therapy, c) investigation of the in vivo molecular mode of action of 8-aminoquinolines, d) characterization of some Trypanosoma and Plasmodium spp.

A new initiative was implemented whereby the multidisciplinary capabilities of three different contractor laboratories and in-house programs on novel chemical synthesis and formulation, in vivo testing in cutaneous and visceral models of Leishmania disease and in vitro mode of action and efficacy studies on identified classes of candidate agents were focused on a common objective of antileishmanial drug development. Special emphasis is placed on purine analogs, allopurinol, allopurinol riboside and WR 6026 development. The antileishmanial activity of purine analogs, transmethylase inhibitors, and WR 6026 derivatives was determined. The comparative activity of drugs against Leishmania in human macrophages and in mouse macrophages was presented and in vivo testing of encapsulated drugs against L.

donovani infected hamsters was initiated. The biochemical mechanisms of ketoconazole against leishmanial promastigote sterol metabolism and of WR 163577 against Trypanosome kinetoplasts were determined. The mechanisms of action of Pentostam against macromolecular synthesis in *Leishmania* are being studied.

A computer program for the processing and analysis of *in vivo* drug testing data was initiated. This program should allow for characterization and referencing of antileishmanial compounds by the completion of FY 1985.

Further testing of topically applied antileishmanial compounds were carried out on the *Mystromys/L. braziliensis panamensis* cutaneous model. Drugs presently used in humans were tested (i.e., pentamide, pentostam, stibophen, and primaquine) and all were found to have some efficacy in this system. Studies on the use of pentostam-containing red blood cells ghosts as a vehicle for drug delivery were continued. Preliminary data concerning drug leakage of ghosts was obtained as well as the improvement of methods of efficacy determination of this preparation against visceral leishmaniasis in the hamster.

Cutaneous leishmaniasis was diagnosed in 5 patients. Parasites were isolated from one of these patients and typed by isoenzyme analysis. It was found to be *Leishmania braziliensis panamensis*. All the patients were treated with one course of pentostam and all post-prescription cultures have been negative. A simple kit to type leishmanial isolates by isozyme analysis was field-tested and continues to be used at USAMRU-Brasilia.

Several aspects of the biology and *in vitro* cultivation of *Leishmania* promastigotes were investigated. These included: (1) investigation of agar plate culture techniques for the detection and enumeration of amastigotes and promastigotes of *Leishmania* from sandflies, (2) comparison of the *in vitro* growth characteristics and requirements of several species and strains of *Leishmania*, and (3) an investigation of the differences between promastigotes from logarithmic and stationary phase cultures in terms of their infectivity for the vertebrate host, susceptibility to lysis by serum (complement), lectin binding to the cell surface, and proteins in isolated membranes and whole cell homogenates.

(**Schistosomiasis**) The schistosomiasis primary topical prophylactic drug screen was transferred to the U.S. Army Medical Research Unit in Brasilia, Brazil, in January 1984. Since that time, 140 compounds have been evaluated as potential antischistosomal topical prophylactics.

An effort is currently underway to develop a test system to evaluate the amount of protection U.S. military field uniform fabric provides against infecting schistosome cercariae. Preliminary data from rodent studies indicate that the Army BDU fabric provides 95% protection and the cotton tropical uniform fabric can provide up to 98% protection from schistosomiasis mansoni. Further studies are being conducted to evaluate chemical impregnation of fabric as a method of providing increased individual protection and to develop a model system to reliably test and predict such protection in the laboratory.

Rodent studies on topical prophylactic protection by WR 34912 and WR 46234 have continued. Both compounds provide 24 hours of complete protection at 0.07% in ethanol (w/v) even after extensive washing with water. At a concentration of 0.1%, WR 34912 provided complete protection for 3 days after 3 hr of washing and WR 46234 was effective for 6 days in preventing schistosomiasis even after 3 hr of water washing. In non-human primate studies WR 46234 provided complete protection for 8 out of 9 Rhesus monkeys for 6 days after a single application of a 1.0% solution of the test compound. The drug was applied to the animal's forearm and washed one hr with tap water before exposure to Schistosoma mansoni cercariae. The one WR 46234 treated monkey which became infected had an infection which was only 5% that of the control monkey.

Studies were initiated to evaluate the potential of using microanalytical detection of urine-excreted schistosome worm metabolites for early diagnosis of acute schistosomiasis.

A complete computer database and analysis system has been established for the antipenetrant drug screen. Programs have been outlined and problems identified in the development of compounds identified as potential prophylactics for advancement to the IND phase of development.

A U.S. patent application has been submitted for the determination of a new use patent for a new class of compounds as topical prophylactics against schistosome cercariae. An extensive manuscript reporting these results has been prepared and is awaiting submission pending JAG guidance relative to public disclosure.

Approximately 542 compounds (representing 1,100 potential test groups) were sent to USAMRU-Brasilia for prophylactic and/or curative antischistosomal testing, including representative compounds from promising classes of compounds with previously identified activity. Increased emphasis is being placed on pre-exposure treatment in prophylactic testing. The computerization of the entire Brasilia data base of biological testing results was completed.

Biochemical Pharmacology

Complete studies on the absorption, distribution, metabolism, and excretion of the candidate antileishmanial drug, WR 6026·2HCl, have been performed. A method to determine plasma levels of WR 6026·2HCl has been developed. Absorption and pharmacokinetic studies of the new candidate antimalarial drug, WR 238605, are currently underway. Methods to analyze the candidate antimalarial drug, artesunate, and its breakdown product, dihydroqinghaosu, in biological fluids are also in progress. Metabolism and pharmacokinetic studies of artesunate are also being performed. A sensitive *in vitro* technique to measure a candidate drug's methemoglobin forming properties has been developed and is being utilized to study structure-activity relationships. The *in vivo* methodology utilized to study methemoglobin formation and disappearance has been significantlly improved upon and is currently being utilized in this laboratory.

Future Objectives:

The production of additional quantities of artemisinin (qinghaosu) for toxicity and efficacy studies will receive a high priority of effort, and the growth and cultivation characteristics of the source plant, *Artemisia annua*, will be investigated. The synthesis of new water-soluble thiosemicarbazones is planned. Attempts will be made to optimize antimalarial, antibacterial and antiviral activities of these compounds.

In vivo and *in vitro* screening of compounds for antimalarial, antileishmanial, antitrypanosomal, and antischistosomal activities will continue in attempts to both identify new and unique classes of antiparasitic agents and to better define efficacy and toxicity information for compounds already so identified. Technology and methodologies for more refined and sensitive testing procedures will continue to be developed. Investigations on the mode of action and interaction of qinghaosu alone and in combination with other antimalarial drugs will be conducted, as well as attempts to induce resistance to qinghaosu and enpiroline with cloned isolates of *P. falciparum*. Patterns of cross resistance to other standard antimalarials will also receive attention.

The data base of radiorespirometrically characterized *Leishmania* parasites will be expanded and the radiorespirometry methodology will be focused on studies for parasite drug sensitivity evaluation and/or molecular mechanisms of actions of drugs undergoing development as antiparasitic agents.

Studies will be initiated to improve the sensitivity, rapidity and specificity of diagnosis of leishmaniasis in U.S. soldiers. Increased efforts will be directed toward testing of topically applied compounds against cutaneous leishmaniasis and to developing and refining model test systems for this purpose. Secondary testing in *Aotus* primate models is anticipated. The organ and tissue sequestration of antimony loaded erythrocytes *in vivo* will be studied. Computer data base programs will be developed for for the analysis of taxonomic information of more than 600 *Leishmania* isolates.

Efforts to develop an *in vitro* "pre-screen" for the identification of potential antipenetration compounds against schistosome cercariae will continue, and secondary testing techniques using ^{75}Se radiolabelled cercariae will be improved. The model test system for evaluating the antipenetration characteristics of chemical impregnated and unimpregnated fabrics will be refined and improved. Increased emphasis will be placed on studies to determine the feasibility of identifying juvenile and adult schistosome metabolites in urine as a diagnostic tool. The technology to do this (GC/MS analysis) is now available. Patent applications for antipenetrant compounds will be pursued. Prophylactic and curative screening of compounds will continue and new developments will be pursued as candidate agents are identified. The recently established and enhanced computer analysis and reporting system will greatly facilitate this function. Research on biochemical mechanisms of cercarial penetration as targets for antipenetration compounds will continue.

Absorption, distribution, metabolism, and excretion studies of WR 238605, artesunate and dihydroqinghaosu, will continue. Further structural identification of metabolites of WR 6026 · 2HCL *in vivo* will be performed and studies on the mechanism of action of this compound with respect to efficacy and toxicity will be reinstated. Mechanistic studies on the role of drug metabolism in new drug design will be initiated.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OG 6765	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY	62770A	3M162770A870	AF	046	WMGE
b. CONTRIBUTING					
c. ~~CONTRIBUTING~~	STOG 82/83 —	6.2/3			

11. TITLE *(Precede with Security Classification Code)*
(U) Exploratory Vaccine Development Against Malaria

12 SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In-House

17. CONTRACT/GRANT	
a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE		
FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	2.0	234
85	2.0	253

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of CD&I
b. ADDRESS *(include zip code)* Washington, DC 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Hockmeyer, W T
d. TELEPHONE NUMBER *(include area code)* (202) 576-3551	d. TELEPHONE NUMBER *(include area code)* (202) 576-3544
21 GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: M	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Weber, J L g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22 KEYWORDS *(Precede EACH with Security Classification Code)* (U) Malaria; (U) Recombinant DNA; (U) Vaccine; (U) RAMI; (U) Genes ; (U) Lab animals; (U) Mice

23 TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Malaria is expected to seriously impede military performance whenever troops are deployed in endemic areas. Since the malaria parasites are becoming increasingly resistant to the drugs used in prophylaxis and treatment, an urgent need exists for a vaccine. The objective of this work unit is to clone and characterize genes coding for malaria parasite antigens as a first step in vaccine production.

24. (U) The approaches used as either to clone all the DNA fragments of the malaria parasit in an E. coli expression vector, and then screen for the desired genes using antibodies whic recognize the products or to prepare radiolabeled DNA probes complementary to parasite mRNA, and use these probes to identify and clone the genes which encode the messenger RNAs.

25. (U) 83 10 - 84 09 In collaboration with scientists at the NIH, the gene encoding the major surface protein of malaria sporozoites was isolated from a parasite DNA expression library. The structure of this gene has been examined in 18 different strains of the parasit with the result that the gene, and hence the protein, varies very little among strains. Both the entire sporozoite protein and short peptide segments of the protein are now being synthesized in large amounts for vaccine testing. About 15 different clones were isolated using DNA probes complementary to mRNA from mature intraerythrocyte parasites. Preliminary characterization of these clones using DNA sequencing and nucleic acid hybridization has revealed a complex pattern of related sequences among the clones. Several of the clones appear to contain portions of the same genes. For FY 85, the portion dealing with leishmaniasis will be reported under a new work unit entitled exploratory vaccine development agains leishmaniasis For technical report, see the Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

PROJECT 3M162770A870 MEDICAL DEFENSE AGAINST INFECTIOUS DISEASE

Work Unit 046 Exploratory Vaccine Development Against Malaria

Investigators:
Principles: CPT James L. Weber, MSC
LTC Wayne T. Hockmeyer, MSC

Associates: SP4 Dale R. Edwards

Problems and Objective:

Malaria is a global health problem. It has been estimated by WHO that there are 250 million cases annually and as many as 1.5 million deaths. In the past, malaria has played havoc with U.S. military forces. There were over one million cases among military personnel in the Civil War, and about one half million in WW II. During the Korean and Vietnamese Wars, malaria, was still a major health problem but was particially blunted through the use of prophylactic drugs. Parasite resistance to drugs is however an increasing problem and is rapidly increasing. It is expected that troops deployed today in endemic areas would face a severe health threat from malaria.

A vaccine for malaria is thus a major goal. Evidence exists that immunity to malaria can be induced is solid. Although human malaria parasites cannot be cultured in vitro in quantities even approaching those needed for vaccination of large groups, using the tools of modern molecular biology it has become possible to clone the genes coding for surface antigens with the purpose of ultimately producing large amounts of antigen for vaccination either by peptide synthesis or by production of the antigen within microorganisms.

Our primary objective, is to clone and characterize parasite genes encoding protective antigens. Since the parasites exist extracellularly in three forms in the mammalian host, the gametocyte, merozoite and sporozoite, and since each form undoubtedly has many proteins on its surface, the problem of finding which proteins if any, would make an effective vaccine is a formidable one. Basic knowledge of parasite molecular biology is urgently needed to help solve this problem.

Progress:

The gene encoding the major protein found on the surface of human malaria sporozoites has been cloned and studied intensively in a joint effort between WRAIR and NIH scientists. This protein, the circumsporozoite or CS protein, currently holds the most hope for a malaria vaccine because irradiated sporozoites will induce immunity when injected into human volunteers and because these protective antibodies react with the CS protein and will block sporozoite invasion of host cells *in vitro*. Our part in the collaborative effort was to insert the recombinant bacteriophage DNA containing the CS protein gene into appropriate host cells for production of sufficient amounts of antigen for the initial immunochemical tests. We also characterized the CS proteins synthesized in *E. coli*, subcloned the CS protein gene fragments into plasmids for ease in study and isolation, and have worked out the first steps in purification of the protein from *E. coli* cells. The CS antigen produced in *E. coli* is stable to boiling, and a considerable purification can be achieved by boiling bacterial lysates and centrifugating out insoluble, denatured material.

For a vaccine to be universally effective, it must protect against all strains of the parasite. To test whether the CS protein gene varies between strains of the parasite, we have analyzed, through the method of nucleic acid hybridization, 18 different strains of parasites from around the world. Our results show that although minor differences exist between strains, overall the gene is surprisingly constant. We have not found anything which should prevent a successful sporozoite vaccine. Immunogeneity studies are underway in animals using synthetic peptides or various constructs of the CS protein produced in bacteria.

Our efforts to clone genes encoding other parasite antigens have been mostly channeled into two approaches. The first approach involves cloning all the genomic DNA from the parasite into an *E. coli* expression vector, and then screening the resulting library with antibodies against parasite protein. This approach was used to clone the *P. falciparum* CS protein gene, and has been successfully used to clone other malaria parasite genes. At WRAIR we have produced one expression library in an *E. coli* plasmid using mixtures of DNA fragments released from the parasite chromosomes by cutting with several different restriction enzymes. Screening of this library with monoclonal antibodies to the CS protein was unsuccessful. More recently, we have nearly completed the construction of an expression library in the bacteriophage vector gt11; in this case the parasite DNA was chopped up with mung bean nuclease.

We hope to begin screening this library soon with monovalent, polyclonal antisera against blood stage parasite antigens.

The second approach involves the use of radioactive DNA probes complementary to mRNA isolated from a synchronized parasite population to select genes encoding large amounts of message from genomic DNA libraries. Using cDNA probes made from mRNA of merozoite cell precursors, we have isolated about 55 DNA clones. Preliminary characterization of these clones indicates that many appear to hybridize to the same large genomic DNA restriction fragments and therefore may be portions of a single gene family. Further analysis of these clones including DNA sequencing is underway.

Recommendations:

1. Genomic DNA expression libraries should be completed and should be screened for as many genes encoding parasite antigens as practical.

2. Characterization of the genes already cloned by using cDNA probes should be completed and published. More genes should be cloned using the cDNA probes from other stages.

3. Some effort should be given to analysis of the genome of the parasite, including one or more of the following: analysis of parasite chromosomes by the new alternating direction electrophoretic technique, gene mapping by biochemical means, analysis of repetitive and transposable elements, and analysis of AT-rich spacer DNA.

4. A postdoctoral fellow should be added to the recombinant DNA lab as soon as possible to increase capabilities.

Published Papers:

1. Dame, J.B., Williams, J.L., McCutchan, T.F., Weber, J.L., Wirtz, R.A., Hockmeyer, W.T., Maloy, W.L., Haynes, J.D., Schneider, I., Roberts, D., Sanders, G.S., Reddy, E.P., Diggs, C.L. and Miller, L.H. 1984. Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite Plasmodium falciparum. Science 225, 593-599.

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1. Weber, J.L. and Hockmeyer, W.T. (1984) Structure of the circumsporozoite protein gene in 18 strains of *Plasmodium falciparum*. (Submitted for publication, Molecular and Biochemical Parasitology).

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OB 6526
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR)636

3. DATE PREV SUMMARY: 83 10 01
4. KIND OF SUMMARY: D.Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM A. WORK UNIT:

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A870	AT	048 WH07
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Field Studies of Rickettsioses and Other Tropical Diseases

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE: 73 07
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORK YEARS	b. FUNDS (In thousands)
84	6.5	100
85	5.0	156

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER (include area code)
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME
U.S. Army Medical Research Unit-Malaysia
b. ADDRESS
Institute for Medical Research
Kuala Lumpur 02-14, Malaysia
c. NAME OF PRINCIPAL INVESTIGATOR
LEWIS, G E JR
d. TELEPHONE NUMBER (include area code)
984155, 984249
e. NAME OF ASSOCIATE INVESTIGATOR (if available)
KELLY, D J
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
HASTRITER, M W

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Leptotrombidium; (U) Mites; (U) Sennetsu Rickettsiosis; (U) Volunteers; (U) Rickettsia tsutsugamushi; (U) Scrub Typhus; (U) Rickettsia sennetsu; (U) Malaysia; (U) BAW I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23 - (U) The goal of this work unit is the military operational dilineation of rickettsial diseases in the Asia-Pacific region with primary emphasis on elimination of scrub typhus as a military medical problem. Specific problems relating to disease detection, prevention, treatment, pathogenesis and epidemiology are being studied.

24 - (U) 1. Field test doxycycline antibiotic regimes in human volunteers as rickettsial disease prophylaxis under military operational conditions. 2. Perform studies to determine etiology, prevalence, and military relevance of febrile illnesses, particularly those of rickettsial etiology, in Peninsular Malaysia and Sabah. 3. Develop a model for the investigation of the pathogenesis of rickettsial agents in host target cells.

25 (U) 83 10 - 84 09 A highly significant association between Rickettsia tsutsugamushi specific cell mediated and humoral immune responses was found in military personnel participating in field doxycycline prophylaxis studies. Entomological risk assessement in the area of operations demonstrated presence of scrub typhus vectors in sufficient quantities to transmit disease. 2. Studies of febrile patients in rural areas confirmed importance of malaria, scrub typhus, and leptospirosis as causes of morbidity in military personnel. Evidence for sennetsu rickettsiosis as a cause of febrile illness in Peninsular Malaysia was found. 3. Umbilical cord derived human endothelial cells were evaluated as an in vitro model of Rickettsia sennetsu pathogenesis. Three manuscripts were published, one is in press and five were submitted for consideration. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sept. 84.

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Project 3M162770A870 MEDICAL DEFENSE AGAINST INFECTIOUS DISEASE

Work Unit 048 Field Studies of Rickettsioses and Other Tropical Diseases

Investigators:

Principals: LTC George E. Lewis, Jr., VC, USA; MAJ Daryl J. Kelly, MS, USA; MAJ Michael W. Hastriter, MS, USA; MAJ Maarten de Vries, RAAMC; MAJ K. Ganzisan, M&DC, MAF; CPT David D. LaBarre, VC, USA; CPT Andrew C. Taylor, RAAMC; Dr. Lim Thuang Seng, Ph.D.

Associates: Miss Elsie Gan, B.S.
Miss Melinda Lee, B.S.

IDENTIFICATION AND ANTIGENIC ANALYSIS OF *RICKETTSIA TSUTSUGAMUSHI* STRAINS ENDEMIC TO THE ASIA-PACIFIC REGION

Problem: To collect, determine the geographic distribution of, and characterize strains of *Rickettsia tsutsugamushi* within the scrub typhus endemic region. Information on the prevalence and distribution of *R. tsutsugamushi* strains is essential to the development of an effective scrub typhus vaccine. Thus far, research in this laboratory has shown that five of the eight prototype strains are predominant in isolates from Peninsular Malaysia, Thailand, Taiwan, the Philippines, Hong Kong, Australia and the islands of the Northern New Hebrides and Santa Cruz groups (15,16,17,18,19,20,21,22). While these areas represent a large segment of the endemic region, isolates from some of the countries on the regions periphery have yet to be obtained and characterized.

Progress: Human, animal, and chigger *R. tsutsugamushi* isolates obtained from Pakistan were partially characterized using the direct fluorescent antibody test. Of the eleven isolates, two were shown to be similar to only the Karp prototype strain of *R. tsutsugamushi* 4 were similar to the TA716 strain alone and 5 demonstrated cross reactivity with both the Karp and TA716 strains.

Collaborative efforts with the U.S. Navy were established for the purpose of obtaining and characterizing *R. tsutsugamushi* isolates from the Mt. Fuji training area in Japan where 56 cases of scrub typhus have been reported in U.S. Marines in the last 3 years. In addition, efforts are proceeding to obtain isolates from other endemic areas in Japan.

Recommendations: Our knowledge of *R. tsutsugamushi* prevalence in scrub typhus endemic areas continues to increase. Efforts to obtain and characterize isolates from Japan and China, particularly those causing disease in U.S. service personnel in Japan, should continue.

A SEROLOGICAL SURVEY OF THE PREVALENCE OF RICKETTSIAL ANTIBODIES AMONG INHABITANTS OF BANGGI ISLAND

Problem: To determine the prevalence of various rickettsial antibodies among sampled residents of Banggi Island, an island situated off the northern coast of Sabah, East Malaysia. Unpublished reports of recent surveys performed by the Institute for Medical Research (IMR), Kuala Lumpur, have documented that the Banggi Island inhabitants have an unusually high incidence of malaria and filariasis. Subsequently the USAMRU-M was asked to assess the importance of rickettsial diseases on the island, and to provide the findings to the Sabah Medical Services for use in the formulation of health care programs for Banggi Island.

Progress: A USAMRU-M scientist accompanied an epidemiological team from both the IMR and the Sabah Medical Services on a field trip to Banggi Island. One thousand one hundred single blood samples were collected on filter paper from school children and villagers inhabiting the island. Filter paper specimens were tested at the USAMRU-M by standard IFA techniques for the detection of *R. tsutsugamushi* and the spotted fever group.

Of the 1,100 specimens collected, to date 225 have been tested for the presence of rickettsial antibodies. An analysis of these results reveal that 4.9% of the population tested demonstrate antibody to the spotted fever group rickettsiae (tick typhus) at titers of ≥ 50, while 1.3% demonstrate specific antibody to *R. tsutsugamushi* (scrub typhus).

Comparing these data with those obtained during the Ulu Kinta (Peninsular Malaysia) survey (manuscript in preparation) we note that the prevalence of spotted fever group specific antibodies is comparable (4.9% versus 4%). Although the populations in these surveys are not strictly comparable because of age and sex differences the results suggest that both scrub and tick typhus are as frequent in Banggi Island as in Peninsular Malaysia

Recommendations: If the samples tested thus far prove to be representative of the total population studied, we will recommend that the causative agent of tick typhus on Banggi Island, and its vector(s), be identified so that feasibility of control measures can be assessed. We will also advise that *R. tsutsugamushi* antibodies be surveyed in at risk populations, such as rural workers, rather than school children and village communities.

INVESTIGATION OF THE ETIOLOGY OF FEBRILE ILLNESS OCCURRING IN A PARAMILITARY BASE

Problem: Malaysian Police Field Force (PFF) personnel operating in northern areas of Malaysia have been affected by a high incidence of febrile disease, and often the cause has remained undiagnosed. The objective of this study was to determine the relative incidence of selected major tropical diseases, particularly those amenable to specific treatment, as a percentage of febrile illness suffered by members of a special paramilitary group. Studies of febrile illnesses performed by USAMRU-M in certain rural areas have demonstrated that a laboratory diagnosis is possible in 63% of febrile cases, and, that as high as 54% of febrile illnesses are of rickettsial, bacterial or protozoal etiology (2). Other studies have also shown the importance of laboratory support in differentiating the major tropical illnesses, and that feedback of laboratory results and epidemiological data, increases clinical diagnostic accuracy (1).

Progress: A clinical laboratory was set up on the paramilitary base and 703 consecutive febrile patients were studied. Results were returned to the medical officer as quickly as possible to aid case management. In all a laboratory diagnosis was made in 128, or nearly 20%, of the 703 cases. Sixty-five (65) patients were confirmed as having malaria, all were *Plasmodium vivax*. There were 24 cases of scrub typhus, 15 cases of leptospirosis, 13 cases of typhoid fever and 13 cases of melioidosis. We also confirmed the results of Berman et al (1), by showing that these major tropical diseases all presented a very similar clinical picture and often required rapid laboratory assistance for differentiation.

It is thought that the reasons that a definitive diagnosis was reached in only 20% of cases rather than the expected 40% reflects our comparatively broad criteria for entry to the study, in that any fever of >37.2 C of any duration was defined as a febrile illness, and the fact that the clinic is easily available and free to the paramilitary forces. Also, the referenced study was conducted among inpatients at district hospitals and therefore could be expected to include a greater percentage of seriously ill patients.

Recommendations: We propose to extend our studies on this population with increasing emphasis an malaria.

CHEMOPROPHYLAXIS FOR HUMAN SCRUB TYPHUS STUDIES

Problem: To determine and compare the efficacy of each of the two different doxycycline regimes for the prevention of scrub typhus, in a military situation. An evaluation of the prophylactic efficacy of doxycycline in the prevention of scrub typhus in military troops would be enhanced by understanding both the prevalence of scrub typhus in mammalian populations and the dynamics of the vector mite populations in areas occupied by troops.

Progress: Two battalions of Malaysian soldiers were randomly and evenly divided into 3 groups and given either doxycycline 200 mg once weekly, plus a placebo once weekly, doxycycline 200 mg twice weekly or a placebo twice weekly. Blood samples were collected before, during and on three occasions after a two month jungle operation. The field trial was completed in July, 1984. Approximately 750 soldiers entered the study, with nearly 600 of these taking the prophylactic medications as prescribed and donating blood on four or more occasions. Serological and hematological testing is expected to be completed by early 1985.

In order to assess the relative risk of exposure to military personnel to the disease hazard of scrub typhus, entomological teams concurrently visited the operational area. The study included trapping of rodents by conventional means and collection of larval vector mites by the black plating method. Mammals were trapped in and around troop bivouac areas in Lembau Klau, Minco Estate, and the Kedau Forest Reserve (Pahang State, Malaysia). Each rodent was bled to enable attempted isolation of R. tsutsugamushi and to obtain quantitative R. tsutsugamushi antibody titers. Seven of the total 19 rodents collected in the three areas had >1:50 titers for R. tsutsugamushi specific antibody. Five of the 7 had R. tsutsugamushi infections as demonstrated by laboratory isolations. Isolations were obtained from 2 rodents demonstrating specific antibody titers <25. In addition, larval mites were removed from each rodent and identified. Sixteen species constituting 5 genera among the family Trombiculidae were represented among the total 934 larval mites. Leptotrombidium (L.) deliense constituted 65% of the total mites collected from the rodents and were found in two of the three areas surveyed.

Black plating was conducted in 8 distinct geographical areas. Black plating was standardized by placing ten 4 1/2 in. x 6 in. black formica plates within a one meter square site, waiting 10-15 seconds, and then examining each black plate for mites. All mites seen were collected from the black plate and placed in vials containing distilled water. After 10 plates were examined at one site, they were never replaced at the same site regardless of the number of larvae collected with the first attempt. An approximate 10 meter interval was maintained between black plate sites to eliminate bias created by overcollecting in smaller foci harboring large populations of mites. Every site surveyed in each area was counted whether mites were collected or not. Great care was taken to keep vials containing mites cool at all times until the larvae were processed by the direct fluorescent antibody technique (FA) for the presence of R. tsutsugamushi (3). A total of 1,534 black plated larvae were identified and 1,421 were examined (dead larvae were identified, but not tested by the direct FA technique) by the direct FA technique.

Data of significant consequence regarding potential troop exposure to *R. tsutsugamushi* are: (1) positive *L.* (*L.*) *deliense* larvae were collected in 5 out of the 8 areas surveyed; (2) the vector species *L.* (*L.*) *deliense* was collected in 100 percent of the areas surveyed; (3) within the 8 areas surveyed, an average of 34.6 percent (213 sites) of the total 616 sites surveyed yielded larvae; (4) fifteen percent of the 213 sites yielding larvae had one or more *R. tsutsugamushi* positive larvae; (5) 4.7 percent of all larvae tested were positive for *R. tsutsugamushi*; and (6) *L.* (*L.*) *deliense* constituted 79 percent of the black plated larvae identified (1,211/1,534).

Based on these data, a soldier's chance of encountering an exposure to one or more larval mites while standing, sitting or lying on the ground at random locations is approximately 1 out of 3. Of this statistic, each has a 15 percent chance of being bitten by an infected *L.* (*L.*) *deliense*.

Recommendations: Upon completion and analysis of the serological and hematological data, this project will be terminated. Findings and recommendations concerning the prophylactic administration of doxycycline will be published for military use.

CROSS MATING OF *LEPTOTROMBIDIUM (LEPTOTROMBIDIUM) DELIENSE* FROM MALAYSIA AND THAILAND

Problem: *Leptotrombidium* (*L.*) *deliense* is considered the most significant vector of *Rickettsia tsutsugamushi* in Southeast Asia. Unpublished data generated during collaborative studies between AFRIMS, Bangkok, Thailand, and this laboratory have shown several morphological differences among *L.* (*L.*) *deliense* collected in Thailand and Peninsular Malaysia. The existence of sibling species among the major vector of *R. tsutsugamushi* could have significant impact on the epidemiological understanding of scrub typhus throughout its distribution. It was the purpose of this study to determine if *L.* (*L.*) *deliense* collected from remote geographical areas are capable of interbreeding and to define their distinct morphological differences.

Progress:

Colony origins. Four groups of L. (L.) deliense collected from distinct geographical areas were utilized during this study. Thailand chigger lines were collected by black plate from a forested area about one mile north of the Sakarat Environmental Research Station Headquarters. All Thailand mites used in this study were derived from two females reared to adulthood. The Malaysian lines were derived from a colony in the 18th generation established in 1970 from the Subang Forest, Selangor state; from F1 wild caught L. (L.) deliense collected in 1983 from Raub, Pahang state;and from F1 wild caught L. (L.) deliense collected in 1983 from the Elmina Estate, Selangor state.

Mating regimen. Deutonymph and adult stadia were fed mosquito eggs three times per week throughout the study. All mites were isolated one per vial during the teliophane stadium to ensure virginity. All adults were less than 70 days old at time of pairing. Males and females were paired for 7 days in snap top vials containing charcoal: plaster-of-paris (1:9). Spermatophores were observed during each pairing and males were removed after 7 days. Females were observed for egg laying for 53 days after removal of males. If egg laying was not observed during this period, females were paired with a second male from their own geographical area and observed for egg laying to ensure their fertility. The fertility of all males was determined after pairing with females from a different geographical area by pairing with a female from the same geographical area. Twenty-three individual Thailand females were paired with Malaysian males (15, 4, and 4 from Subang, Raub, and the Elmina Estate, respectively). The males from Subang, Raub, and the Elmina Estate were each paired with females from the long established Subang colony following pairing with the Thailand females. A reciprocal cross was conducted by pairing 4 sibling Thailand males with Subang females. Unfortunately, two of the males died following pairings. One male was paired with 3 individual females, a second and third male was each paired with 2 individual females, and a fourth male was paired with a single female resulting in a total of 8 pairings. Proof of fertility was conducted for each male except for the fourth male which died before pairing with a Thailand female.

Morphological comparisons. A series of all progeny derived from back crossing congeneric species was mounted on glass microscope slides with Hoyer's media to provide specimens for morphological comparisons to elucidate systematic variations and/or differences in Thailand and Malaysian L. deliense.

The 23 Thailand females failed to lay eggs when paired with Malaysian males. All of the 23 Thailand females which failed to lay eggs produced viable eggs when mated to males from their own geographical area. Nine of the original 23 Malaysian males mated to Thailand females proved to be fertile. Likewise, none of the 8 Malaysian females layed eggs when paired to Thailand males. Three of the 8 Malaysian females laid viable eggs when back crossed to Malaysian males. Morphological comparisons remain to be completed.

Recommendation: To be formulated after results are completed (to include morphological studies).

ELECTROPHORETIC STUDY OF TROMBICULID (L. DELIENSE, L. FLETCHERI, L. ARENICOLA) MITES INFECTED WITH RICKETTSIA TSUTSUGAMUSHI

Problem: To develop an electrophoretic technique for use in differentiating the sex and species of mites, as well as their infection status. Within the past 10 years, the use of electrophoretic techniques to separate isoenzymes, with subsequent detection by histochemical staining, has become widely accepted. This technique has been extensively used in studying the genetics of a number of disease vectors, in particular mosquitoes. The availability of infected and uninfected colonies of L. arenicola and L. fletcheri and uninfected L. deliense has presented an opportunity to apply this procedure.

Progress: The separation of several enzymes from newly-emerged adults was performed using starch-gel electrophoresis. Both infected and uninfected male and female mites were tested. In addition, comparative studies were done with those L. fletcheri mites caught in the field by black-plating and those extracted from wild caught rodents. Differences in the patterns of two enzymes, phosphohexoisomerase and phosphoglucomutase, have been identified in studies involving adult L. arenicola and L. deliense. In similar studies of L. fletcheri, both enzymes were polymorphic in the materials examined. Both gene-enzyme systems were represented by two alleles.

Recommendations: Studies involving the electrophoretic separation of isoenzymes of Trombiculid mites have been completed. Useful and interesting data have been acquired and a manuscript is in preparation.

CYTOGENETIC STUDIES OF TROMBICULID MITES

Problem: To perform chromosome studies on mites to determine if there is a detectable karyotypic difference between infected and uninfected mite species and sexes. Cytogenetic data are available for some mites in the suborders Mesostigmata, Astigmata, Cryptostigmata, and Prostigmata (10), but no information is available for mites of the family Trombiculidae. Included in this family is the genus Leptotrombidium, which contains the vectors of scrub typhus. In Malaysia, the 3 principal vectors are L. deliense, L. fletcheri, and L. arenicola (26).

Progress: L. deliense of generation 15, L. fletcheri of generations 37-39, and L. arenicola of generations of 25-27 were studied to determine karyotypic differences between infected and uninfected mite colonies (11,14). Each colony had been maintained continuously under laboratory conditions (9) and originated from a single uninfected female. For these studies, cytologic preparations were made using the method outlined by Kanda (7) with slight modifications. All dissections were performed in a small drop of hypotonic sodium citrate solution; harvested tissues were placed in a colchicine solution and incubated at room temperature for 10-20 min. Tissues were then transferred into a small drop of 45% acetic acid on a siliconized coverslip for 20-30 sec and a drop of lacto-aceto-orcein was added and mixed with the fixative. A clean slide was placed onto the siliconized coverslip, the preparation air dried and the coverslip sealed with nail polish. Phase-contrast was used for karyotypic differentiation.

Larvae, protonymphs, deutonymphs, tritonymphs, and adults of the 3 species of Leptotrombidium were studied for karyotypic differences. The brain, salivary glands, excretory tubules, and reproductive organs were harvested from all developmental stages except larvae and protonymphs. In the last 2 stages, aside from the salivary glands, a preparation was made from a mixture of other tissues, since they were unrecognizable as distinct entities.

Chromosome like structures were observed in the cells from the reproductive organs of all stages but were too small to differentiate as distinct entities except in adults. One to 6 day old adults were tested, and varied numbers of cells containing chromosomes were detected in all ages, with the best results obtained from 2 day old specimens. All but 1 of the successful preparations were from males. The mitotic chromosomes of *L. deliense* and *L. fletcheri* showed 2*n* = 14, while those of *L. arenicola* showed 2*n* = 28. The chromosomes were small and appeared somewhat similar, with lengths ranging from 1.4 - 1.6 um for *L. deliense*, 1.3 - 1.9 um for *L. fletcheri*, and 1.1 - 1.5 um for *L. arenicola*. A small satellite chromosome (0.35 um in *L. arenicola* and 0.8 um in *L. deliense* and *L. fletcheri*) was infrequently seen in some cells from all species.

Only 1 adequate slide from a female *L. deliense* was successfully prepared. Chromosomes from this preparation did not differ in number or size from those observed in the males of the same species. Two to five females from each line of the three species were kept separate and observed for parthenogenesis during each generation. To date, there has been no parthenogenesis observed.

The probable vectors of scrub typhus belong to 1 group of the subgenus *Leptotrombidium*, which is referred to as the *L. deliense*-group (26). However, 2 members of this group, *L. deliense* and *L. fletcheri*, differ from each other both taxonomically, i.e., essentially in the number of dorsal setae (25) and ecologically, i.e., habitat difference (24). On the other hand, there is an overlap in habitats of *L. deliense* and *L. arenicola*, primarily in the coconut plantations along the coastal areas. The number of dorsal setae is also similar in *L. deliense* and *L. arenicola*. However, the scutum of *L. arenicola* is consistently smaller than that of *L. deliense* or *L. fletcheri*. In this study, in which the chromosomes of the 3 vector species were compared, we found that *L. deliense* and *L. fletcheri* appear to be similar (2*n* = 14).

The chromosomes appear to be small, dotlike, and variable in size, but those of *L. arenicola* seem to be the most constant. More descriptive identification of the chromosomes was not possible, but studies using banding techniques with Giemsa and other stains might help to differentiate the karyotypes.

Recommendations: This study has been completed and a manuscript has been submitted.

TRANSMISSION ELECTRON MICROSCOPY STUDY OF *R. TSUTSUGAMUSHI* INFECTED AND NONINFECTED *LEPTOTROMBIDIUM (L.) FLETCHERI* MITES FED ON *R. TSUTSUGAMUSHI* INFECTED ICR MICE

Problem: To conduct a transmission electron microscopy study of imbibed *R. tsutsugamushi* in infection resistant trombiculid mites. The uptake and destiny of *R. tsutsugamushi* in noninfected chigger lines fed on *R. tsutsugamushi* infected mice has not been studied ultrastructurally by TEM techniques. An understanding of the biological events leading to maintenance of infected chigger foci (islands) is necessary to facilitate understanding the epidemiology and the potential control of scrub typhus.

Progress: Rickettsiae were not observed in *R. tsutsugamushi* free *L. (L.) fletcheri* fed on a *R. tsutsugamushi* infected host when examined at 3, 8, 48 and 88 hours post attachment. Rickettsia-like organisms were numerous throughout naturally infected larval tissues at 3, 8, 48, and 88 hours post-engorgement. Specifically, rickettsiae were seen in salivary glands, muscle tissues, nervous tissues, gastric tissues, hemocytes, secretory cells, epithelial cells, epidermal cells, and were found extracellularly in the hemolymph. The esophagus appears to be chitinized from the buccal cavity postad to the area exiting the fused supra and subesophageal ganglia (brain). The columnar epithelial cells lining the chitinized esophagus passing through the brain have numerous rickettsiae, many of which lie directly against the chitin surface. The posterior gut expands into a large lumen at 3 and 8 hours, but becomes diminished by 48 hours and inapparent at 88 hours post detachment. The large gut lumen is lined with epithelial cells directly beneath the microvilli projecting into the lumen. Rickettsiae were not seen within the lumen of the esophagus or hind gut. During the early phases of engorgement, hemocytes and large metabolic spherules are sparsely distributed. However, at 48 hours the posterior-lateral coelomic cavity becomes congested with hemocytes containing electron dense granules and is dominated by metabolic spherules throughout the coelomic cavity. The moulting process is underway at 8 hours although salivary glands, muscles and chitin have not undergone much observable disassociation. Salivary glands are heavily infected with rickettsiae at 3 and 8 hours and they contain numerous mitochondria and concentically arranged patterns of rough endoplasmic reticulum calyxing into alveolar tubules. At 48 hours the large laterally bilobed salivary gland tissues have almost completely disassociated. The rickettsia are bound in groups within large membrane bound structures. At 88 hours the areas once occupied by the salivary glands are inundated by the large metabolic spherules. No rickettsiae were noted within the metabolic spherules. Many extracellular rickettsiae can be seen at 88 hours.

Within muscle tissues, rickettsiae were seen most frequently at the terminal ends of muscle bundles in areas rich in mitochondria. Of particular interest are the numerous close associations of rickettsiae and mitochondria in all types of tissues. Often mitochondria nearly, and in one observed case, completely envelope the rickettsia in which they were associated.

Polar microtubular structures are present in some elongated rickettsia-like organisms. The dense outer laminar membrane appears similar to the shorter rickettsia-like organisms which are characteristic of the typhus group of rickettsia. These elongated rickettsia-like organisms appear indistinguishable from those found in L. (L.) arenicola larvae and adults by Wright, Hastriter, and Robinson, 1984 (29). The bipolar microtubular structures are also indistinguishable.

The presence of morphologically similar elongated rickettsiae with microtubules found in both L. (L.) fletcheri and L. (L.) arenicola is highly suggestive of either/or (1) common evolution of host/parasite, (2) organism is a pleomorphic variation of R. tsutsugamushi necessary in the in vivo life cycle of the mite host.

Recommendations: Specific immunoperoxidase staining of the chigger tissues to distinguish *R. tsutsugamushi* from other rickettsia like symbionts must yet be performed. Direct FA testing of chigger frozen sections are currently being conducted as well as further TEM examinations of these chigger tissues. Upon completion of these procedures this study will be finished and a manuscript will be prepared.

ESTABLISHMENT OF A *RICKETTSIA TSUTSUGAMUSHI*-INFECTED *LEPTOTROMBIDIUM DELIENSE* MITE COLONY.

Problem: To establish a *Rickettsia tsutsugamushi* infected colony of *Leptotrombidium deliense* and perform bionomic comparisons between the old and a newly established *L. fletcheri* colony. The USAMRU-M maintains a *R. tsutsugamushi* infected colony of *L. fletcheri* and *L. arenicola*, but not *L. deliense*. *Leptotrombidium deliense* is considered the most significant vector of scrub typhus in Southeast Asia. Laboratory bionomic data constituting only one publication on an infected line of *L. deliense* has been generated, that being from a laboratory in India. Sex ratios published therein are significantly different from *L. arenicola* and *L. fletcheri* in this laboratory. These bionomic inconsistencies warranted evaluation.

Progress: In efforts to establish an infected *L. deliense* colony, larval chiggers were collected by black plating in designated sites containing 3-5 year old oil palm trees. Direct FA tests of individual mites confirmed the presence or absence of infected mite foci at each designated oil palm site. Additional collections were made at sites where *R.tsutsugamushi* positive mites were identified. The mites collected from these sites were fed individually on adult ICR mice. Mites shown to transmit scrub typhus were to be propagated as candidates for establishment of an infected colony. *R.tsutsugamushi* positive mite foci were identified at three areas in the Elmina oil palm estate. Fourteen percent (13/92) of the *L. deliense* larvae collected were positive for *R. tsutsugamushi* by the direct FA technique. Subsequently, three collections constituting 219 larvae were made at these positive foci, 182 of these mites were singly fed on and recovered from mice. Spleen suspensions of the chigger fed mice were passed in naive adult ICR mice using standard techniques so as to ascertain transmission of R. tsutsugamushi from those mites to the chigger fed mice.

Recommendations: Efforts to establish an infected L. deliense colony should continue. These efforts should be of a collaborative nature with the IMR since there is no acarologist present or planned for the USAMRU-M.

ISOLATION CHARACTERIZATION AND EVALUATION OF R. TSUTSUGAMUSHI MARKER ANTIGEN

Problem: Our lymphocyte transformation (LT) and macrophage inhibition factor (MIF) assays use both membrane (Frac. 4) and soluble (Frac. 3) antigens which are prepared by a modification of the French pressure cell procedure described by Dasch *et al*.[27] However, due to the crude nature of these antigens, an undesirable degree of serotype cross reactivity often occurs in both LT and MIF assays. Thus, if we are to have a better understanding of the duration of both homologous and heterologous immunity to R. tsutsugamushi, it is necessary to (a) identify and isolate specific antigen(s) tnat could be used in the LT assay which would differentiate initial from subsequent homologous and heterologous infections, (b) isolate a specific immunogen that could be used to obtain high affinity antibody in animals for use in an early antigen detection assay and (c) isolate rickettsial protein fraction(s) that would elicit effective CMI responses in selected laboratory animal models.

Progress: Groups of Balb/c mice, immunized subcutaneously with either crude membrane or the soluble fraction of Gilliam strain rickettsial antigen, were challenged with a potentially lethal 1000 50% mouse lethal doses (MLD) of Karp strain R. tsutsugamushi. Sixty percent of the mice receiving soluble antigen survived the subsequent challenge and only twenty percent of the mice receiving membrane antigen survived an identical challenge. Analysis using polyacrylamide gel electrophoresis showed four thin and two thick bands of protein were separated from the membrane antigen and only three protein bands were isolated from the soluble antigen.

Recommendation: The antigenicity of each protein band should be studied in the LT and MIF assays. The appropriate fraction(s) should be qualitatively recovered and characterized. This project will be completed by early 1985.

CULTIVATION AND STUDY OF *RICKETTSIA TSUTSUGAMUSHI* IN HUMAN ENDOTHELIAL CELLS

Problem: To examine the growth characteristics and pathologic effects of *Rickettsia tsutsugamushi* in cultured human endothelial cells.

Progress: Cultured human endothelial cells (HE), both *R. tsutsugamushi* infected and uninfected, were examined by light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). In addition, the growth rate of the rickettsiae was determined by titration of infected cell samples (taken at specific time intervals), in mice. These results were compared with those from concurrent experiments using an established cell lines.

Cells derived from human umbilical cord veins were confirmed to be of endothelial origin by morphological and immunological criteria. Morphologically, the HE cells were as described by Jaffe, et al. (6). Particularly prominent were the nuclei and nucleoli, as well as the "blebs and pits" found mostly in the perinuclear region and peripherally, in uninfected cells. Using TEM, Weibel-Palade bodies similar to those described by Jaffe (6), were occasionally seen in the cytoplasm. The indirect FA test for factor VIII antigen verified the morphological findings. L-929 and MRC-5 cells were negative for factor VIII antigen.

Light microscopic examination of Giemsa-stained HE cells harvested 1 hr post-infection showed that few cells (approx. 5%) contained rickettsiae. At 4-6 days post-infection, there was a considerable increase; an average of 85% of the cells were infected by day 6. The rickettsiae were mainly present in the perinuclear region of the cytoplasm; heavily infected cells remained intact. On day 8, the infected HE cells were becoming granulated and about 20% of the cells had disintegrated. Giemsa-stained preparations showed numerous rickettsiae in the cytoplasm of each cell and also extracellularly. At 10 days, the infected cells began to release from the surface of the flasks - both individually and in clumps. By day 12 post-infection, virtually all of the cells had released from the surface.

Infected L-929 and MRC-5 cell control cultures went through the same sequence of infection. The L-929 cells began releasing from the surface on day 6, while MRC-5 cell monolayers were intact until day 8 post-infection. Infected MRC-5 cells tended to release as a sheet of cells, rather than as single cells or small clumps. By day 8, greater than 99% of each of the three cell types were infected with *R. tsutsugamushi*.

Direct FA testing of infected HE, L-929, and MRC-5 cells confirmed that the organisms seen were *R. tsutsugamushi*.

Mouse titrations of cell suspensions of infected HE, L-929, and MRC-5 cell monolayers were performed. Growth rates in the HE and MRC-5 cells are similar from days 4 through 8. *R. tsutusugamushi* attained higher titers in HE as compared to MRC-5 cells; titers in L-929 cells were considerably lower than in HE cell suspensions.

In SEM studies numerous rickettsiae were seen protruding, both horizontally and vertically, from the surfaces of infected HE, MRC-5, and L-929 cells. Chaining of rickettsiae was seen, both intra- and extracellularly. HE cells were often seen which were heavily infected with *R. tsutsugamushi* and remained intact. Neither MRC-5 nor L-929 cells appeared, by SEM, to become as heavily infected as HE cells. The morphology of the rickettsiae protruding from the cell surface was indistinguishable from one cell type to another.

TEM examination of *R. tsutsugamushi*-infected HE, MRC-5, and L-929 cells revealed no differences in the ultrastructure of the intracellular rickettsiae. No rickettsiae were seen in the nuclei of any of the cell types; however, both intact and degenerating rickettsiae were seen in the cell vacuoles. Intracytoplasmic rickettsiae escaped from the host cells by budding through the cell membrane, as previously seen by others (4,12,28). The freed rickettsiae were coated with a layer of host cell membrane. Occasionally, intracytoplasmic rickettsiae were seen that had what appeared to be two layers of host cell membrane.

Recommendations: This project has been completed.

EVALUATION OF TROMBICULID MITES AS POTENTIAL VECTORS OF *EHRLICHIA SENNETSU*

Problem: To determine if *E. sennetsu* can be transmitted by trombiculid mites. Although *E. sennetsu*, the etiologic agent of a well defined clinical entity, sennetsu rickettsiosis, is known to occur only in Japan, recent collaborative studies between USAMRU-M and the University of Illinois suggest the presence of *E. sennetsu* like agents in Peninsular Malaysia. The vector of *E. sennetsu* in the Pacific Asiatic region is unknown. A potential vector, one which has been proven to be the vector of another species of rickettsia is the trombiculid mite. The object of this investigation was to evaluate *Leptotrombidium fletcheri*, a species of mite closely related to one commonly found in Japan, as a potential vector for *E. sennetsu*.

Progress: Adult ICR mice were inoculated with 1,000 50% mouse lethal doses of *E. sennetsu*. Whole blood of these mice was titered every 2 days through day 18 post inoculation. The period of peak rickettsemia which varied between of 4.5-5.0 50% mouse lethal doses/0.2 ml of whole blood occurred between days 10-16 post inoculation. Both *R. tsutsugamushi* infected *L. fletcheri* mites and uninfected mites were tested for *E. sennetsu* prior to feeding. Mites were mass fed, 40/host, on rickettsemic mice. *E. sennetsu* was not isolated from or demonstrated by the indirect fluorescent antibody (FA) technique in, any of the mite stadia, nor were the organisms transmitted by the F-1 generation in either mites naturally infected with *R. tsutsugamushi* or mites free of *R. tsutsugamushi* infection. The predominant sex among the group of adult mites free of *R. tsutsugamushi* infection was male. Of the 31 adults individually inoculated for attempted isolation, only 7 were females and 24 were males. Proportionately, 143 of the total 158 adults test by the direct FA/indirect FA techniques were males, while the remaining 15 adults were females.

AVAILABLE

Recommendations: Results from this study suggest L. fletcheri cannot acquire E. sennetsu from infected mice. Recent studies in the United States demonstrate an antigenic relationship between E. sennetsu and Ehrlichia canis, a canine pathogen transmitted by ticks (5,13). Recommend this project be continued into the in collaboration with the IMR which has a recently established tick colony. In addition, this study should be repeated using the L. arenicola and L. deliense colonies available.

CMI RESPONSE OF MICE TO INFECTION WITH RICKETTSIA TSUTSUGAMUSHI

Problem: To develop and evaluate a reliable assay for the measurement of cellular immune responses to R. tsutsugamushi infection. Cell mediated immunity (CMI) has been demonstrated to be a major factor in acquired resistance to R. tsutsugamushi infection in mice. The need for meaningful CMI assays for both the mouse and the monkey, as well as the transfer and application of this technology to human scrub typhus studies, for which there are no published data,requires the development of lymphocyte transformation (LT) and macrophage inhibition factor (MIF) assays. These assays could be used to measure the onset and longevity of cellular immune responses to infection with R. tsutsugamushi. In addition, the relevance of each assay in predicting immunity to reinfection with homologous or heterologous strains of R. tsutsugamushi could be determined.

Progress: The membrane and soluble fractions of French pressure cell treated, tissue culture grown strains Karp and Gilliam of R. tsutsugamushi were prepared and used in this study. Both Karp and Gilliam infected Balb/c and C3H/He mice were solidly immune to back-challenge with Karp throughout a 1 year study period. Transfer of spleen cells from Karp and Gilliam infected mice to their respective syngeneic normal recipients resulted in protection against a subsequent potentially lethal Karp challenge of the recipients. These CMI transfer experiments proved that spleen cells from infected mice were able to transfer protection against a lethal Karp challenge in naive recipients for at least one year.

Mitogenic responses to PHA were suppressed in Karp infected Balb/c and C3H/He mice from days 11-14 days to day 28. Positive LT responses to homologous and/or heterologous antigens were detected in 80% of the Karp infected mice on day 7 and then were suppressed until day 28. This transient suppression affected not only *in vivo* lymphocyte response to homologous and heterologous rickettsial antigens but also affected antibody response to unrelated antigens such as sheep red blood cells. At 365 days post-infection, postiive lymphocyte responses were still detected in all groups of mice. From 7 to 44 days post-infection (PI), supernatants derived from spleen cell cultures from Karp and Gilliam infected mice demonstrated an ability to inhibit *in vitro* macrophage migration. Furthermore, at the time of peak infection (day 14-21) supernatants from cells incubated without antigen were also found to have MIF activity. This activity however, was not enhanced when antigen was added to the cell cultures.

Delayed type hypersensitivity (DTH) was detected at 7 days PI in 40% of Balb/c mice infected with either Karp or Gilliam. However, DTH was not detected in Karp-infected C3H/He mice until 32 days PI. By day 60 PI, less than 40% of all mice tested have positive DTH response. The MIF assay is clearly superior to the footpad swelling test for the detection of DTH.

Recommendations: The mouse study has been completed and manuscripts are in preparation.

INVESTIGATION OF THE SEROEPIDEMIOLOGY OF *EHRLICHIA SENNETSU* IN MALAYSIA

Problem: To determine if sennetsu rickettsiosis, a human febrile illness resulting from infection with *Ehrlichia sennetsu*, is present in Malaysia. Infectious mononucleosis (IMN) is believed to be caused by the Epstein-Barr virus (EBV) and is characterized by fever, lymphadenopathy, headache, pharyngitis, and malaise. The disease classically effects young adults resulting in considerable and prolonged morbidity, but only very rarely does it prove fatal. Sennetsu rickettsiosis produces an almost identical clinical picture to this in western Japan, and in fact, is often clinically diagnosed as IMN. This is of practical importance as sennetsu rickettsiosis responds well to tetracycline therapy (8,23) and IMN does not.

Progress: In collaboration with Dr. M. Ristic of the University of Illinois, sera from various population groups have been assayed for the presence of *E. sennetsu* specific antibodies. Isolation of the suspected organism has been attempted from patients demonstrating a high antibdy titer.

1. Serology: Over 2,000 sera from various population groups have been tested by indirect fluorescent antibody techniques. Prepared antigen slides for use in both USAMRU-M and in Dr. Ristic's laboratory are currently supplied by Dr. Ristic, but USAMRU-M will be producing antigen independently in early 1985.

2. Isolation: Two *E. sennetsu* like organisms have been isolated at Dr. Ristic's laboratory after mouse and cell culture inoculation. These are yet to be fully characterized.

Results show that the prevalence of antibody to *E. sennetsu* in the general Malaysian population to be very low, while in a group of patients clinically suspected to have infectious mononucleosis the prevalence is 38%. In the only PUO group so far examined with a clinical disease consistent with sennetsu rickettsiosis the antibody prevalence is 52%. This is certainly strong epidemiological evidence for the occurrence of *E. sennetsu* infection in Malaysia.

Recommendations: We believe that there is strong evidence that *E. sennetsu* infection occurs in Malaysia, and that infection with this agent results in a disease clinically similar to infectious mononucleosis. We propose to continue our studies of this disease with emphasis on both isolation at USAMRU-M, of the organism from patients at USAMRU-M, and on clinical studies of patients with sennetsu rickettsiosis like illness.

EVALUATION OF THE HUMAN ENDOTHELIAL CELL AS AN *IN VITRO* MODEL THE STUDY OF THE PATHOGENESIS OF *EHRLICHIA SENNETSU*

Problem: To delineate the growth characteristics and pathologic effects of *Ehrlichia sennetsu* in cultured human endothelial (HE) cells to further our knowledge of rickettsiae/host interactions in sennetsu rickettsiosis.

Progress: A system for culturing umbilical cord derived human endothelial cells was adapted for the propagation of *Ehrlichia sennetsu*. Cultured HE cells, both *E. sennetsu* infected and uninfected, were examined by light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Cells were confirmed to be of endothelial origin by morphological and immunological criteria. Light microscopic examination of Giemsa stained cells showed few (approx. 5%) infected cells up to 10 days post *E. sennetsu* inoculation. By day 14-16 an average of 95-100% were infected. By day 16 infected cells were rapidly releasing from the flask surface. Indirect fluorescent antibody staining of infected HE cells confirmed that organisms seen were *E. sennetsu*. Distribution patterns of organisms within the cells were similar to those seen using Giemsa stain. SEM studies revealed numerous pleomorphic ehrlichiae protruding from the surface of infected HE cells. TEM examination of the cells revealed pathogen induced cytopathic changes at the ultrastructural level. Ehrlichiae of variable sizes were located individually and in clusters enclosed within membrane lined vacuoles. Each organism within these clusters demonstrated a plasma membrane and a distinct outer cell wall. Organisms ranged from small highly electron dense bodies to larger more diffuse structures.

Recommendations: These preliminary findings suggest that the cultured endothelial cell, which for certain ehrlichial and rickettsial diseases represents the putative "target" cell, provides an optimal environment for the growth of *E. sennetsu* and is a suitable *in vitro* model for study of the cytopathic effects of this human pathogen. Further studies are recommended to include comparisons of growth rates of *E. sennetsu* in HE cells and other established cell lines, as well as comparison of growth rates in primary HE cells vs. multiply passed HE cells derived from the same source.

INCIDENCE OF *R. TSUTSUGAMUSHI* INFECTION AND CMI RESPONSES IN MALAYSIAN ARMY VOLUNTEERS

Problem: The objectives of this study are: (1) to compare the lymphocyte transformation (LT) assay with indirect fluorescent antibody (FA) in its ability to

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detect the presence of previous exposure to *R. tsutsugamushi* in the volunteers, (2) to determine the immunity of volunteers to *R. tsutsugamushi* infection, (3) to determine the applicability of the LT assay as a means of early detection of scrub typhus and, (4) to determine the ability of the assay to differentiate between a primary *R. tsutsugamushi* infection and a reinfection.

Progress: This project is nearing completion. Analysis on some of the data recently completed indicates a highly significant association between LT responses and indirect FA responses among the 205 volunteers tested. The results indicated 36 out of the 205 volunteers had antibody to at least one of the 3 prototype strains (Karp, Gilliam and Kato) of *R. tsutsugamushi*. Furthermore, 33 of the same 36 indirect FA positive volunteers also showed positive LT responses which constituted 92% of the indirect FA test positive volunteers.

Recommendation: The completion of this study will help answer many questions regarding human scrub typhus and immunity. Human scrub typhus studies will give way in 1985 to malaria projects having a priority higher than does scrub typhus.

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*Underlining indicates the individual who presented the paper.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

DA OB 6525	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
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10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A870	AN	049 WWQ2
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/3			

11. TITLE *(Precede with Security Classification Code)*
(U) Schistosomiasis, Malaria and Leishmaniasis Studies in Brazil

12. SUBJECT AREAS
0603 Biology 0613 Microbiology 0615 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
73 07	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	2.5	100
85	3.0	105

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
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Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
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20. PERFORMING ORGANIZATION
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b. ADDRESS
Brasilia, Brazil
c. NAME OF PRINCIPAL INVESTIGATOR
HEMBREE, S C
d. TELEPHONE NUMBER *(include area code)*
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f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
BOSWORTH, A B
g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
HOCH, A L

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Lab Animals; (U) Mice; (U) Brazil; (U) Schistosomiasis; (U) Malaria; (U) Leishmaniasis; (U) Entomology; (U) Epidemiology; (U) RAM I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Quantify malaria transmission dynamics and establish an epidemiological data base in an area potentially to be used for vaccine testing. Determine the unknown reservoir host(s) and vector(s) of Leishmania b. braziliensis in an endemic area. Conduct primary screening of agents for prophylactic or therapeutic activity against schistosomiasis. Study schistosomiasis epidemiology and control in an endemic area. This research is of military importance.

24. (U) Initiate malaria epidemiology and vector biology studies in an area of the Brazilian Amazon which is under development. Continue reservoir host studies and initiate search for vector(s) of cutaneous leishmaniasis by various capture and examine methods. Use self-sustaining mouse-snail-schistosome culture system for primary drug screening. Continue control efforts by various means and monitor transmission of schistosomiasis in an endemic area.

25. (U) 83 10 - 84 09 A malaria epidemiological study site was selected in the southwest Amazon. Initial population census and mapping are complete, and baseline medical data have been collected on about one fourth of the population. Known vector species and their breeding sites have been found. Anopheline production and dispersal studies continued in Labrea, Amazonas. A small-area ULV control study was conducted on the Ituxi River. Studies to determine the vector(s) of cutaneous leishmaniasis were initiated. Apparent non-human pathogens have been found in man-biting species. Isolates of human leishmanial pathogens have been made from wild mammals in an endemic area, and appear to be L. mexicana amazonensis. Screening of anti-cercarial penetration agents was initiated and screening for chemotherapeutic and prophylactic activity continued. Activity was detected in 13 compounds. Mollusciciding resulted in 98 percent reduction of snail population in a schistosomiasis endemic area. For technical report see WRAIR annual report 1 Oct 83 - 30 Sep 84.

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PROJECT: 3M162770A870 MEDICAL DEFENSE AGAINST INFECTIOUS DISEASES

Work Unit 049: Schistosomiasis, Malaria and Leishmaniasis Studies in Brazil

Investigators: LTC Stephen C. Hembree, Ph.D; LTC Anthony B. Bosworth, Ph.D; MAJ Alfred Lynn Hoch, Ph.D; Dr. Aluizio R. Prata, M.D.; Dr. Reynaldo Dietze, M.D.; Mr. Norman Peterson

PROBLEMS AND OBJECTIVES

Malaria, leishmaniasis and schistosomiasis continue to pose a threat to American military personnel who are or who might have to be stationed in the Middle East, Africa, the Far East, the Caribbean or Latin America. These diseases also inhibit development and cause great human misery, a potential source of political unrest, in many of the developing countries where they are found. These diseases are highly accessible to study through a cooperative arrangement between the University of Brasilia and the Walter Reed Army Institute of Research.

Malaria is a major and growing public health problem in Brazil. Since 1975, the number of cases reported per year has almost tripled. More than 300,000 cases were reported in 1983, an increase of 35% over the previous year. The vast majority of these cases occurred in the sparsely settled Amazon Region, where the availability and distribution of medical services mitigate against full reporting. Thus, these figures probably represent a gross underestimate. With the opening of road nets in the Amazon and the active promotion of migration into the region by the Government of Brazil, many areas are under development and are being settled by people from other parts of Brazil. The highest occurrence of malaria in Brazil is in these developing areas, especially in the southwest Amazon, in the state of Rondônia.

Control of malaria in the Amazon is complicated by numerous factors including limited resources, transportation difficulties, the high dispersion of the population, the rudimentary and open design of the housing and the behavior of both man and mosquito. Use of residual DDT as a control method, to which the major vector species are still physiologically susceptible, has had only limited success. Many of the dwellings are not fully enclosed. People frequently stay outside their dwellings during the early evening hours, the time at which the vector species feed most actively. Additionally, DDT has been shown to have a repellent effect on some populations of the primary vector.

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The malaria situation is degenerating, as indicated by Ministery of Health statistics, and practicable control measures are desperately needed. Possibilities include the development and proper use of new drugs, a malaria vaccine, and/or new vector control strategies. Design and optimum implementation of any control measure under the condition of limited resources requires a detailed understanding of the transmission biology and dynamics of malaria under the extant conditions. Only the gross aspects of malaria epidemiology in the Amazon are known. The objective of our field malaria studies is to provide basic information on vector biology and malaria transmission dynamics upon which alternative malaria control strategies can be based. These studies also provide a matrix within which new technology relative to malaria investigation and control can be developed and/or tested.

Technology for the production by recombinant DNA methods of a vaccine against falciparum malaria has recently emerged, and candidate vaccines will soon be availabe for testing. Evidence suggests that immunity to malaria may be strain specific to a considerable degree. This raises questions about the number of antigens that must be represented in a vaccine and the degree of cross protection provided against one strain by antigens from another. We know practically nothing about strains of malaria from an immunological standpoint. Fundamental questions, such as whether or not one or more than one strain exists in a given area, and what is the range of distribution of any given strain, have not been answered. It may be possible to use growth inhibition assays in microcultures to study strain specificity of immunity. The objective of our malaria immunology project is to use growth inhibition assays to address fundamental questions such as those above about malaria strains.

As far as is known, cutaneous leishmaniasis is a sand fly borne disease with wild mammal reservoirs. It is widespread in Brazil, and the mucocutaneous form of the disease, commonly caused by *Leishmania braziliensis braziliensis*, is frequently encountered in some areas south of the Amazon. The disease is difficult to diagnose in its early stages. Culture for diagnosis and study of some strains of the etiologic agent can not be done reliably. There presently is no way to determine the probability that an infection with *L. b. braziliensis* will metastasize to the mucocutaneous form. Therefore, the philosophy of treatment has been that all infections must be treated vigorously. Treatment is of long duration, involves the use of toxic drugs and sometimes has to be repeated because of ineffectiveness and/or relapse. It is difficult to confirm cure. The mucocutaneous form of the disease is potentially hideously disfiguring and may have a fatal outcome. The many days required for therapy and the detailed follow-up required to confirm cure would cause an extreme burden on military medical facilities, if large numbers of troops become infected. The grossly disfiguring effects of advanced mucocutaneous leishmaniasis would horrify and have a negative psychological effect on troops serving in an endemic area, unless they could be given genuine assurance that the disease is preventable or treatable. We are presently unable to prevent this diseases, and the reservoir host(s) and vector(s) of L. b. braziliensis are unknown. Thus, well focused control strategies can not be implemented against them. It is highly relevant to the development of locally effective transmission control methods that the vector(s) and animal reservoir(s) for the disease be identified and their accessibility to control measures determined. Leishmaniasis research at USAMRU/Brasilia has the objective of determining the vector(s) and the reservoir host(s) of cutaneous leishmaniasis at a study site where active transmission is taking place, concomitantly with habitat modification, in government encouraged agricultural developments and where metastasis to the mucocutaneous form of the disease is common. Access to patients in an area where active transmission is taking place provides an opportunity to investigate all aspects of the disease.

The mode of transmission of schistosomiasis is such that troops moving through or stationed within endemic areas could be expected to experience a high level of exposure and infection. The distribution of infectious foci is spotty, and there is no way to identify them other than laborious and detailed search for cercariae in water and/or snail hosts. There currently is no single drug that is totally satisfactory for treatment of schistosomiasis. More importantly, there is presently no prophylaxis against the disease, and infected troops, even when treatable, already represent a loss of combat strength and a burden on medical resources. Research on schistosomiasis at this laboratory has the primary objective of detecting, in a primary screening system, compounds with prophylactic and/or therapeutic activity against the disease. Access to field study areas with infected human populations and active transmission provides an opportunity for testing new anti-schistosomiasis technology and methods for investigating the disease and the biology of its transmission.

PROGRESS

Malaria. The addition of a Brazilian physician to our staff in April, 1984, permitted the initiation of a malaria epidemiology project. This project is intended to provide background information on the transmission dynamics of malaria in a human population, a geographical area, and a demographic situation potentially suitable for future field testing of malaria vaccine and/or new therapeutic or prophylactic agents. Since malaria is a complication frequently accompanying development in the Amazon, a collateral objective is to study the changes in malaria transmission during the development process. The project will make extensive use of what has already been learned of the biology, movement and reproductive behavior of *Anopheles darlingi* in previous and continuing studies in an adjacent part of the Brazilian Amazon.

Selection of a study site was based on a number of biological, demographic, geographical and political considerations. These combine to assure the presence of measurable malaria transmission in an accessible area with a demographic situation characteristic of the Amazon under development and in a political environment that assures longitudinal acceptance and support of the project. Field trips to and malaria prevalence surveys on population totaling over 1600 persons were conducted at Fortaleza do Abunã, Ariquemes and Costa Marques in Rondônia, in the southwest Amazon. Species of known malaria vectors were found in each area, and both *Plasmodium falciparum* and *Plasmodium vivax* were present in each area. However, Fortaleza do Abunã is not accessible during part of the rainy season, and the population was less than 500 people. Ariquemes, on the newly paved part of the Trans-Amazon road net between Cuiabá, Mato Grosso, and Porto Velho, Rondônia, already is well developed, with a population of 60,000 - 70,000 people in the study area. Remigration from this area was common, with one observation reporting that about 30% of the people in one area remigrate during a one year period. Ariquemes appeared unsuitable in view of our collateral objective in the project of recording the changes in malaria transmission as settlement and development take place.

Costa Marques is on the Guaporé River, which forms much of the geographical boundary between Bolivia and Rondônia. It is at the end of a road being built and scheduled for completion in November, 1984, from the Cuiabá - Porto Velho axis to the Guaporé Valley. No population concentration resides on the Bolivian side of the border. The area has been relatively isolated and extensive development has not yet begun. An asphalted landing strip will be ready by the rainy season of 1984-1985, guaranteeing year round access. The present population of the area appears to be about 3,000 people, including a dispersed rural population. Local acceptance and political support at all levels has been generously manifested. Laboratory space, examining rooms and living quarters have been provided. Local census and mapping are almost complete and baseline medical and demographic date have been collected on almost 800 persons. A firm commitment to the project has been made by the Universidade de Brasília, the Núcleo de Medicina Tropical, and the government of the State of Rondônia.

Although all laboratory equipment, reagents and trained staff are present, the initiation of the study of strain specificity of immunity to falciparum malaria has been delayed by the inavailability of suitable study material. Being sought are sera and parasites from long term residents of several study areas who are apparently at immunological equilibrium with the local strain(s) of falciparum malaria; i.e., persons with circulating *P. falciparum* parasites without clinical malaria. Location of such subjects has not been possible, because, until recently the University has had no field malaria project within the contexts of which these subjects could be sought. It is anticipated that only small numbers of such subjects will be found, because malaria in Brazil is characteristically unstable, and therefore, subclinical malaria is relatively uncommon. Also, the high mobility of the human population in the developing Amazon mitigates against assurance of a high level of integrity in the geographical representativeness of isolates. However, the present study area, because of its degree of isolation, may provide access to malaria isolates that are genuinely representative of the areas in which they are found. To date, only four isolates of malaria from subclinical cases have been made. The integrity of these is low, because the patients were not under observation long enough to determine if the cases might not have been pre-symptomatic infections rather than asymptomatic. Subclinical falciparum malaria has been identified in some subjects in the Costa Marques area, and these cases are being observed.

Malaria vector biology studies in the vicinity of the city of Labrea, Amazonas, continued. Longitudinal observations of seasonal prevalence and spatial distribution of larval and adult stages are being correlated with malaria occurrence within the city of Labrea and with the annual hydrological cycle. Six species of *Anopheles (Nyssorhynchus)* were collected. These were *An. darlingi*, *An. braziliensis*, *An. triannulatus*, *An. nuneztovari*, *An. oswaldoi*, and *An. evansi*. To provide high quality material for detailed taxonomic study, larvae were collected and reared to adults in order to acquire larval and pupal skins corresponding to preserved adult specimens.

At Floresta, a field study site two days by boat up-river from Labrea, small area ultra-low volume (ULV) insecticide spraying was attempted for the control of adult *An. darlingi* and to elucidate certain aspects of movement and resting behavior. The project was a cooperative effort with SUCAM, the malaria control agency of the Brazilian Ministry of Health, which provided the materials and the pesticide applicator. The insecticide, fenitrothion, was applied as a ULV mist from a backpack sprayer such as could be used to protect small military encampments. Spray was applied on the vegetation surrounding the clearing containing the dwellings at Floresta. Effects of two treatments were evaluated using mark, release and recapture methods. Baseline man-biting collection data were acquired two consecutive days before the first treatment.

Mosquitoes were released at the study site before and after treatment. Two releases of marked mosquitoes were made at 300m distance after the second treatment. Recapture rates indicate that mosquitoes resting in the clearing or on vegetation surrounding it were killed. These could have been parous females that had returned to the clearing for a subsequent blood meal. Among these could have been infected females. Also, non-parous females arriving at the clearing, seeking their first blood meal, successfully or not, could have been killed. A high influx of new arrivals continued, as indicated by high capture numbers of non-marked mosquitoes in man-biting collections on the night following treatment and by a high recapture rate of mosquitoes released 300m from the treated area.

A single generation of *An. darlingi* was naturally acquired from the offsprings of field collected specimens on two separate occasions. This represents the first successful mating of *An. darlingi* in the laboratory in three years of trying to colonize this species, the primary malaria vector in much of South America, including the Brazilian Amazon.

An. darlingi females fed readily through an artificial membrane made of a synthetic rubber film with a nylon support screen. About 91% of 130 female mosquitoes engorged on blood recovered from malaria cultures. Hematocrit of the blood was adjusted to 12%. Sixty percent of the fed mosquitoes were still alive after 17 da. Only 10% sucrose was provided as food after the blood meal.

A human-use protocol for studies on the susceptibility of known and suspected species of Brazilian *Anopheles* to human malaria parasites has been drafted and is being circulated for local comment before submission to WRAIR. A thirty contact-hour course entitled "Arthropods and Mollusks of Medical Interest" was presented to graduate physicians at the Núcleo de Medicina Tropical.

Leishmaniasis. Studies to determine the wild mammal reservoir host of *Leishmania braziliensis braziliensis* in the vicinity of Três Braços, Bahia, continued. Field laboratory and animal care facilities were made available to the project in a new health post recently constructed in Três Braços. All mammals collected were necropsied in the Três Braços facility, and tissue specimens (skin, spleen and liver) were inoculated into hamsters. Arthropod ectoparasites were collected from all specimens and sent to specialists. Mammalian study materials were sent to the Division of Mammals, Smithsonian Institution in a cooperative study of the mammals of the Brazilian coastal forest region, an area poorly represented in their collections.

In the Três Braços area 68 nights of trapping (3,678 trap nights) yielded 113 mammals of 14 species. An outbreak of *L. b. braziliensis* in the vicinity of Corte de Pedra, 50km by road from Tres Braços, presented an opportunity to examine mammals captured near homes in which patients with new infections resided, in an area where extensive active transmission was taking place. A house was rented in Corte de Pedra, and arrangements were made to use the local health post as a field laboratory. Seventeen nights of trapping (1,352 trap nights) resulted in the capture of 84 mammals of 15 species.

Isolates of leishmanial organisms originating from three spiny rats (*Proechimys iheringi denigratus*), collected in tall forest habitat near Três Braços, were determined by analysis of 9 isoenzymes to be closely related to *L. mexicana amazonensis*. The parasite was distinguishable from *L. mexicana ssp*. from the state of Goiás, Brazil, *L. m. mexicana*, *L. m. pifanoi*, *L. m. garnhami* and *L. m. aristedesi*. Two additional isolates, as yet unidentified, have been made from the spiny rat, one from the same habitat as the above and another from secondary forest habitat 11km way.

With the addition to our staff of an additional entomologist in March, 1984, field and laboratory studies were initiated to determine and study the biology of the sand fly vectors of cutaneous leishmaniasis in the Três Braços and Corte de Pedra areas. A field surveillance program was initiated to collect sand flies by a variety of collecting techniques in the various sylvatic, agricultural, peri- and domestic environments represented in the endemic areas. Epidemiological observations support the hypothesis that peri-domestic transmission occurs, and current concentration of effort is in that habitat. Collected specimens are identified to determine the local fauna and its habitat specificity and are dissected to determine the presence of the leishmanial parasite. Nine species have been identified, with *Lutzomyia* (*Lu.*) *whitmani* forming 95% of the peri-domestic anthropophilic fauna. A flagellate parasite has been recovered and cultured from *Lu. whitmani* at a peri-domestic collection site in Corte de Pedra. The parasite grew well in two types of media normally used to culture leishmanial parasites. However, it failed to infect hamsters and probably is not a *Leishmania*. Its identity remains in question. This find questions the identity of past isolates made from this sand fly and assumed to be *Leishmania* based on culture growth and morphology. The taxonomy and life cycle of the parasite are being studied in two sand fly species.

Attempts were successful to colonize anthropophilic species from the study area in order to facilitate studies of vector potential. The most common man-biter in the area, *Lu. whitmani*, is in culture and five other species were reared in the laboratory from eggs recovered from gravid, wild caught females. These species were *Lu. davisi*, *Lu. flaviscutellatus*, *Lu. hirsutus*, *Lu. fischeri* and *Lu. aerozai*. *Lu. flaviscutellatus* is a recognized sylvatic vector of *L. m. amazonensis* in the Amazon Region of Brazil.

The Leishmania laboratory at the University of Brasília hosted an extended technology transfer TDY by MAJ Patrick McGreevy, Department of Parasitology, WRAIR. This trip resulted in numerous constructive observations and recommendations. Methods used at WRAIR for the primary isolation of Panamanian *Leishmania* were inadequate for the primary isolation of *L. b. braziliensis*, and it has been recommended that the Brazilian methods be installed at WRAIR. The taxonomy and distribution of *Leishmania* in the Americas is confused. Some 150 leishmanial isolates from people, animals and sand flies, representing new species, drug resistant strains and pathogens of mucosal disease were transferred to the WRAIR cryobank for further classification. On the recommendation of MAJ McGreevy, a study was initiated in the endemic area to determine if the high doses of Glucantime, selected empirically to prevent metastasis to the mucocutaneous form of the disease, could be reduced to the minimal dose necessary to cure skin lesions without increasing incidence of mucocutaneous disease. A visit to the Brazilian Jungle Warfare Training Center in Manaus confirmed that training operations in the jungle frequently resulted in infections with cutaneous leishmaniasis. A high level of command empnasis and a regime of preventive medicine measures had reduced transmission. In his trip report MAJ McGreevy asked the highly relevant question of whether or not the US Army had adequate supplies of drug and sufficient technical expertise to cope with an outbreak of cutaneous leishmaniasis of significant size.

Schistosomiasis. The Brazilian physician added to our staff in April, 1984, was assigned primary responsibility for the schistosomiasis drug screening program and will continue making epidemiological observations at a study area in which he had conducted an integrated schistosomiasis control program. Transfer from WRAIR to USAMRU, Brasília, of the protocol for screening externally applied agents to prevent cercarial penetration reflected increased emphasis on prevention of this disease. Screening of injected and ingested agents for prophylactic and curative activity continued. In the anti-penetrant protocol 129 agents were screened, of which two showed activity by the criteria of the protocol. In the primary mortality test, designed to detect prophylactic activity in injected or ingested agents, 349 agents were screened, and activity was detected in four compounds by the criteria of the protocol. In the primary curative test 1,052 compounds were screened, of which seven were active by the criteria of the protocol. Screening protocols were assigned by Division of

Experimental Therapeutics, WRAIR, and the screened compounds are identified to USAMRU, Brasilia only by bottle number. Data are forwarded to Division of Experimental Therapeutics, WRAIR, for further action.

At field study sites in endemic areas, treatment trials were initiated to evaluate the efficacy of a single dose of Oltipraz and the efficacy of Praziquantel and Oxamniquine given together in a single treatment against Brazilian *Schistosoma mansoni*. Other observations were made on the regression of hepatosplenomegaly following treatment with Oxamniquine. Treatment success was evaluated by Kato-Katz fecal exams 6 and 18 mo after treatment. After 6 mo, regression was found in 12.1% and after 18 mo in 38.9%. Follow-up studies and retreatment were initiated on 400 patients who had not responded to an initial treatment with oxamniquine. Three attempts to infect sentinal mice by placing them in local waters were unsuccessful. Up to 80% of the rodent *Cavia aperea* was found to be infected with *S. mansoni* in some foci in the study area, before snail control was initiated. Retreatment of local waters with Bayluscide resulted in 98% reduction in vector snail populations. The effect of snail control on the prevalence of *S. mansoni* in *C. aperea* is currently being studied.

RECOMMENDATIONS

1. Implement the malaria epidemiology project at Costa Marques, Rondônia, to include longitudinal studies of local anopheline populations.

2. Continue the anopheline surveillance program in Labrea, Amazonas.

3. Continue attempts to colonize *An. darlingi*.

4. Quantify susceptibility of potential malaria vectors to *Plasmodium vivax* and *Plasmodium falciparum* by feeding laboratory reared females on informed, consenting malaria patients in hospitals at Costa Marques and Labrea and by using a membrane feeding technique with cultured falciparum malaria.

5. Continue efforts to detect the vector(s) and reservoir host(s) of agents causing cutaneous leishmaniasis in Bahia and study their biology with the objective of designing control methods against them.

6. Quantify susceptibility of potential sand fly vectors to leishmaniasis by feeding laboratory reared insects on informed, consenting subjects with active disease under medical supervision.

AVAILABLE

7. Provide colonized sand flies and local strains of leishmaniasis to other laboratories developing specific monoclonal antibody assays, dot enzyme - linked immunosorbent assays (DOT-Elisa) and DNA probe agent detection methods.

8. Continue the anti-schistosomiasis agent primary screening program, with continued emphasis on detection of prophylactic potential.

9. Continue monitoring efficacy of integrated schistosomiasis control efforts in Caatinga do Moura.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA OB 6530	2. DATE OF SUMMARY 84 10 01	*REPORT CONTROL SYMBOL* *DD-DR&E(AR) 636*
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A870	AN	050 WW06
b. CONTRIBUTING				
c. ~~CONTRACT/GRANT~~	STOG 82/83-6.2/3			

11. TITLE *(Precede with Security Classification Code)*
(U) Vaccine Development in Trypanosomiasis

12. SUBJECT AREAS
0603 Biology 0613 Microbiology

13. START DATE 73 09	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		84	8.0	319
c. TYPE	d. AMOUNT	85	8.0	259
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME U.S. Army Medical Research Unit-Kenya
b. ADDRESS *(include zip code)* Washington, D.C. 20307-5100	b. ADDRESS Box 401 USAMRU-K APO New York 09675
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR WHITMIRE, R E
d. TELEPHONE NUMBER *(include area code)* (202)-576-3551	d. TELEPHONE NUMBER *(include area code)*
21. GENERAL USE FINA	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* NJOGU, A
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* OSTER, C N

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Lab Animals; (U) Goats; (U) Cattle; (U) Monkeys; (U) RAM I; (U) Volunteers; (U) Leishmaniasis; (U) T. brucei; (U) Immunity; (U) Trypanosomias

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The objective of this program is to develop an effective, practical vaccine against African Trypanosomiasis, useful to both military and civilian agencies. Related benefits include acquisition of knowledge pertaining to trypanosome immunity, host response and pathology of infection. There is a requirement for these studies which should provide a basis for rational development of a vaccine for this disease which would constitute a serious hazard for military personnel operating in the endemic area.

24. (U) Experiments conducted at WRAIR and in Kenya have demonstrated that experimental animals can be successfully immunized with irradiated trypanosomes. Rodents, cattle and monkeys can be rendered completely resistant to a challenging infection of T. rhodesiense.

25. (U) 83 10 - 84 09 During this period the investigators confirmed that there was a significant antigenic shift in the 1980-81 outbreak. Two new zymodemes were identified by isoenzyme assay. Polyvalent sera from the 1970's failed to neutralize either new zymodeme. Epidemiologic and treatment record analysis was completed and is being processed. The African Trypanosomiasis human treatment center received its first patients and work on evaluation of standard treatment regimens was begun. An experimental compound WR 163557 continues to be evaluated in the goat model against T. brucei infection. Classification of Leishmania isolates by cellulose acetate electrophoresis was perfected. A cryobank of Leishmania isolates was established. Three species of sandflies were colonized and are being studied for taxonomic and ecologic characterization. Non-invasive techniques for the diagnosis of human visceral leishmaniasis were developed and are being refined. Maintenance of Rift Valley Fever virus during interepizootic periods has been defined by the discovery that eggs of Aedes lineotopennis found in the soil of dombos (depression in the earth that flood only in periods of excess rainfall) contain virus. Epizootics of Rift Valley Fever has been definitely linked to periods of surplus rainfall in Kenya. For technical report see WRAIR Annual Progress Report 1 October 1983 - 30 September 1984.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

PROJECT 3M162770A870 MEDICAL DEFENSE AGAINST INFECTIOUS DISEASE

WORK UNIT 050 VACCINE DEVELOPMENT IN TRYPANOSOMIASIS

INVESTIGATORS:

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PROGRESS IN TRYPANOSOMIASIS RESEARCH

Epidemiology Survey

Twenty five (25) cases of Human African Trypanosomiasis (HAT) were reported through August 1984. Most of these cases have been diagnosed by the detection center at Magunga in the Lambwe Valley. This facility was established and is staffed by USAMRU-K local national technicians in order to have a diagnostic capability in the Lambwe Valley and thereby reduce transportation requirements for movement of samples and individuals suspected of having the disease.

Human African Trypanosomiasis Therapy Project Alupe

A laboratory capable of performing basic hematology, biochemistry, and parasitology analyses and that can support a 20 bed in-patient clinical research ward has been established. This included importation of all instrumentation, reagents and consumables as well as retraining of Government of Kenya laboratory technicians and laboratory assistants.

The patient care facility was set up and functional in December 1983. Seventeen patients were cared for using WHO drug dosage recommendations with intensive clinical and laboratory monitoring that is intended to provide baseline experience for future toxicity, efficacy, and pharmacokinetic studies.

Tsetse Control Program

The Veterinary Department, Ministry of Agriculture, Government of Kenya initiated a tsetse control program in July 1984 which consisted of spraying insecticide around the periphery of the Ruma National Park in the Lambwe Valley. Subsequent ground spraying and bush clearing within the park game park is now in progress. Only two new cases of HAT have been reported since August 1984..

A new focus of HAT on the shore of Lake Victoria, west of the Lambwe Valley has remained relatively dormant as only one case has been reported in 1984.

Trypanosomiasis Analysis

Two hundred twenty (220) *Trypanosoma brucei* spp. stocks isolated between 1969 and 1983 from the Lambwe Valley were characterized by isoenzyme electrophoresis using 12 enzymes. Twelve different zymodemes of *T.(T.) b. rhodesiense* were isolated from patients during the 13 year period and identical stocks were also found in cattle, reedbuck (*Redunca redunca*) and tsetse flies (*Glossina pallidipes*). Two new zymodemes not found before 1980 predominated in the 1980 outbreak in man. Fourteen of fifty-six Trypanosome isolates from cattle belong to man-infected zymodemes. (This study done in collaboration with W. C. Gibson, Kenya Trypanosomiasis Research Institute).

Polyvalent antisera prepared against *T. brucei rhodesiense* stocks isolated during the 1970's failed to neutralize many *T. b. rhodesiense* isolates obtained in the outbreak in 1980. Over 50% of cattle sampled in the Lambwe Valley had neutralizing antibody to a predominant zymodeme of *T. b. rhodesiense* in 1980. Preliminary evidence suggests a relationship between zymodeme and serodeme classification.

Treatment and follow-up of Human African Trypanosomiasis Patients

In 1980, HAT patients were treated with Melarsoprol (Mel B) only if they presented with central nervous system (CNS) symptoms. Cerebrospinal fluid (CSF) was not analyzed. Under these conditions, 51 individuals were treated with Suramin and 11 with Mel B. Of the 51 patients receiving Suramin, 25 relapsed between three and twenty-six months after treatment. One Mel B treated patient subsequently relapsed. The 25 suramin relapsed patients were treated with Mel B and most have been followed for two years with no further relapses. The one Mel B relapsed patient was given a second course of Mel B which appears to have been curative.

After documentation of the high relapse rate, spinal fluid analysis at presentation was initiated with marked success. Only 3 of 45 subsequent patients treated with Suramin with normal spinal fluid findings have relapsed. On the other hand, many more patients are being treated with Mel B because of abnormal CSF findings on admission. Toxicity of Mel B in patients has not been a serious problem as described in other geographic areas. This may be due to the gradually incremented and extended administration of the drug, however, in the past Mel B has been used by GK medical personnel at increased concentrations in patients from the Lambwe Valley with little evidence of toxicity.

Model for Human African Trypanosomiasis

Goats develop CNS involvement with *Trypanosome brucei* within 2 months of infection. The model has been used to assess the effect of Suramin on CNS infection. It has generally been accepted that Suramin does not penetrate the CNS in sufficient strength to be curative. Single injections of Suramin (50 mg/kg) cure most CNS infections in the goat model. There appears to be no toxicity with this level in the goat, however, the World Health Organization suggests a maximum dose of 20 mg/kg (up to 1 gram) be administered to man in a single injection. Whether an increased dosage would be effective in CNS involvement in man will require further investigation.

This model has also been used to test the prophylatic effect of a bisquinaldine compound (WR-163,557). This drug provided protection for as long as 180 days at both 25 mg/kg and 100 mg/kg. However, mice and goats challenged at 270 days after drug administration developed patent infections and died.

PROGRESS IN LEISHMANIA RESEARCH

Biochemical Characterization of Kenyan Leishmania Isolates and Cryobanking

The use of cellulose acetate electrophoresis (CAE) to characterize and identify Leishmania isolates is currently expanding. To date all wild animal and sandfly isolates in the Nairobi Leishmania Bank (NLB) have been identified. As three human cutaneous manifestation of Leishmania occur in Kenya, i.e. *L. major*, *L. aethiopica*, and *L. donovani* (post kala azar dermal), CAE is also used routinely on all human cutaneous isolates. Plans are currently under way to use the CAE technique to identify all strains/isolates currently in the Leishmania collection. This bank presently has 248 isolates for which appropriate documentation and methodology in record keeping and cryopreservation techniques have been applied. This complies with the World Health Organization criteria for a Leishmania Reference Center. Recent communications with WHO have indicated that they are anxious to have a WHO sponsored Leishmania Reference Center established in Africa and that the Kenya Medical Research Institute/USAMRU-K laboratories could serve as the nucleus for such an effort. In this vein efforts are being made to computerize the data on isolates currently held and equipment has been ordered to facilitate such an expansion.

Animal Model(s)

A search for new animal models is being pursued. The evaluation of the disease process in other animals is being conducted in an effort to elucidate the parasite's behavior in man and reservoir animals.

An initial study to determine the susceptibility of nonhuman East African primates for visceral leishmaniasis was conducted in conjunction with the Institute of Primate Research, a division of the National Museums of Kenya. The first series of experiments was conducted using the Sykes' monkey, *Cercopithecus mitis*; vervet monkeys, *Cercopithecus aethiops*, olive baboon, *Papio cynocephalus* and bush babies *Galago crassicaudatus*. These early experiments demonstrated the Sykes' and vervet monkey to be the most promising laboratory hosts. As a result of these experiments, more indepth studies of both species are currently underway. Twelve vervet monkeys are under investigation to determine the clinical/pathological response to experimental *L. donovani* infections and to study their humoral and cell mediated immune responses during the course of the disease. Five were infected I.V. with 3×10^7 amastigotes/kg and five received 3×10^7 promastigotes/kg I.D. Two animals served as controls.

During the course of previous primate experiments, it was observed that 5 of 5 monkeys inoculated intradermally with promastigotes developed acute lymph node infections on the appendages in which they were originally infected. However 4 of the 5 monkeys involved self-healed after 4 months infection and only one went on to develop visceral leishmaniasis as detected via a positive splenic aspirate. It was felt that this development of lymphadenopathy and the self cure may be similar to the epidemiological pattern found in areas where kala azar occurs. That is, 20-30% of the population are positive by serological tests; yet only 1-2% have any history of clinical disease. We feel that these lymph node infections could account for the subclinical cases found in association with epidemiological surveys. To further evaluate this hypothesis, ten Sykes' monkeys were inoculated ID with L. donovani promastigotes.

In a series of experiments to determine parasite uptake by the reticuloendothelial tissue in Balb/c mice when stationary phase promastigoes were used as a subcutaneous (SQ) inoculum it was determined: (1) that following foot pad inoculations the skin of the foot and the popliteal lymph nodes become parasite positive within five days, (2) Liver and spleen become positive after approximately 20 days.

These early experiments lead to the conclusions that: (1) subcutaneous inoculations of stationary phase promastigotes appear to favor early lymphatic uptake of parasites to the draining lymph nodes, this may be preceded or accompanied by localization of parasites within SQ tissue. (2) Lymphatic uptake to drainage lymph node sites may be an inoculum dose and/or volume-dependent phenomenon. (3) The dynamics of parasite uptake/replication at peripheral RES/dermal sites may have epidemiological implications such as prepatent period disease transmission via parasite availability to sandfly vectors.

An experiment in which both inoculum volume and parasite numbers have been varied is currently in progress. It is planned that the results of this experiment and the use of natural transmission (laboratory infected sandflies) will help elucidate this aspect of the natural epidemiology of the disease.

Mongoose Experiments:

To date several field trips and the collection of large numbers of samples from various animals have failed to disclose the reservoir host(s) for visceral leishmaniasis in East Africa. Over 500 canidae have been examined and only 2 have been postive for parasites which have been typed as L. donovani. Several thousand rodents have been collected and a number of isolates made, however, all such isolates have been identified as L. major. Two earlier reports, one from the Sudan in the 1950's and one from Kenya more recently mentioned isolates from both mongoose and genet cats. Unfortunately in neither case were the isolates typed as to species identification.

A protocol in collaboration with the Department of Mammalogy, National Museums of Kenya, has established a means of acquiring various species of carnivores such as mongoose and genet cats. To date 14 dwarf mongoose, *Helogale parvula* have been collected. After the determination of clinical base line values, these animals will be given a Kenyan strain of *L. donovani* at a dose of 6×10^8 amastigotes/kg of body weight. These animals will be killed at 30 day intervals and their tissues cultured and examined histologically.

If these animals prove susceptible, then wild animals will be trapped in a natural foci of human kala azar to determine their role in the natural epidemology of this disease. It is planned that other species of mongoose and genet cats will be examined in a similar manner.

Treatment of Visceral Leishmaniasis

Since 1983, all newly diagnosed patients with visceral leishmaniasis have been enrolled into a prospective, randomized protocol comparing 4 dosage regimens of sodium stibogluconate: 10 mg/kg once daily for 30 days; 20 mg/kg once daily for 30 days; 15 mg/kg twice daily for 30 days; and 15 mg/kg twice daily for 15 days. In the first 18 months of the study, 142 patients were enrolled. Twelve percent of the patients treated for only 15 days did not respond to therapy, compared to 2, 0 and 0% of the other 3 groups ($P = 0.025$). These data suggest that 15 days of treatment, even with a high dosage of sodium stibogluconate (30 mg/kg/day) is inadequate. New patients will no longer be enrolled in this protocol, but all patients presently enrolled will be followed for 12 months to determine the rates of relapse in the 4 groups.

Patients treated with sodium stibogluconate developed abnormalities on their electrocardiograms (ECG) during 51% of their treatment courses. The frequency of abnormalities were related to both the total daily dosage and the duration of treatment. Most abnormalities were minor and disappeared quickly after treatment was completed. However, 7 patients treated with 30 mg/kg/day, or more, developed prolongation of their QT intervals, and one patient treated with 60 mg/kg/day died suddenly after developing QT prolongation. Sodium stibogluconate is, therefore, safe when used in doses less than 30 mg/kg/day. If higher doses are used, ECG's should be monitored. If QT prolongation occurs, sodium stibogluconate should be withheld until the QT interval normalizes, and then reintroduced at a lower dosage.

Electron photomicrographs of amastigotes in the spleen of patients before and during treatment with sodium stibogluconate demonstrate that this treatment causes autolytic degeneration of the parasites. However, the kinetoplast was well preserved, even in amastigotes in advanced stages of degeneration, suggesting that sodium stibogluconate has a different mode of action than pentamidine, which affects the kinetoplast primarily.

Incidence of Parasitemia in Patients with Kenyan Visceral Leishmaniasis

Twenty Kenyan patients with visceral leishmaniasis diagnosed by the demonstration of parasites in their splenic aspirates were examined for the presence of parasites in their peripheral blood. Smears, cultures or hamster inoculations detected parasitemia in 11, 10 and 6 patients respectively. Overall, parasitemia was detected by at least one method in 15 patients with higher parasite densities in their spleens. We are currently investigating the possibility of diagnosing visceral leishmaniasis by testing peripheral blood samples for parasite specific antigens using monoclonal antibodies or parasite specific DNA using specific DNA probes.

Viable Amastigotes in the Nasal and Pharyngeal Secretions of Patients with Visceral Leishmaniasis

Nasal and pharyngeal secretions were collected from 12 patients with proven visceral leishmaniasis before the start of treatment. Amastigotes were found in 2 nasal smears, and promastigotes were cultured in modified Schneider's medium (containing 5-fluorocytosine) from the nasal secretions of 4 patients and the throat secretions of one patient. Overall, 42% of the patients had parasites in their nasal secretions, and 8% in their throat secretions. The presence of viable parasites in patients' secretions raises the possibility of direct person-to-person transmission, and may explain the intrafamilial clusters of disease that are frequently encountered in Kenya. Furthermore, if a more sensitive assay for the presence of parasites in the nasal secretions is developed, it could obviate the need to perform a splenic aspirate.

Treatment of Patients with Visceral Leishmaniasis Unresponsive to Sodium Stibogluconate

Ten patients were treated at the Clinical Research Center (CRC), Kenya Medical Research Institute after having failed to respond to multiple previous courses of sodium stibogluconate. Nine had been previously treated elsewhere, and 4 had concurrent pulmonary tuberculosis. No clinical or immunological differences were found between these patients and patients who responded to initial treatment with sodium stibogluconate. Six of these patients eventually responded to very high doses of sodium stibogluconate (20 mg/kg three times per day) suggesting that the strains of *L. donovani* infecting these patients were somewhat, but not completely, resistant to the drug. Five other unresponsive patients responded to the combination of sodium stibogluconate and allopurinol. We are currently enrolling patients who have failed to respond to an initial course of sodium stibogluconate, or have relapsed after this treatment into a protocol which randomly assigns them to receive either sodium stibogluconate alone or sodium stibogluconate plus allopurinol to determine if the combination is synergistic in these difficult patients.

Other Parasitic Diseases in Patients with Visceral Leishmaniasis

One hundred twenty-four patients with visceral leishmaniasis were examined for the concurrent presence of malaria and schistosomiasis. None of the patients with visceral leishmaniasis had schistosomiasis and only one had malaria. In contrast, of 69 patients seen with splenomegaly not due to leishmaniasis, 35% had schistosomiasis and 25% had malaria. The reasons for the reduced prevalence of other parasitic disease in patients with visceral leishmaniasis is unexplained, but is unexpected because these patients have severely depressed cellular immune response and are susceptible to secondary infections with bacteria and viruses.

Heat Treatment of Cutaneous Leishmaniasis due to *Leishmania aethiopia*

Two patients with cutaneous leishmaniasis caused by *L. aethiopica* were treated by applying heat with a circulating water bath. The skin temperature at the site of the lesion was raised to 40 to 41 C for 12 hours a day for 4 weeks. Both patients' lesions healed and have remained healed for over one year. Previous experience at the CRC had suggested that treatment of *L. aethiopica* with high doses of sodium stibogluconate (20 mg/kg twice daily) was effective. However, 2 of 3 patients who initially responded to this treatment have relapsed within the past year.

PROGRESS IN RIFT VALLEY FEVER RESEARCH

Isolation of Rift Valley Fever Virus From Reared Adult Male and Female Aedes Linetopennis Collected from an Artificially Flooded Dambo

Because of the failure of seasonal rains, dambos did not flood naturally and a decision was made to attempt to artifically flood a dambo. By aerial survey a suitable dambo was located near a river from which water could be pumped. A 2½ inch petrol powered water pump capable of pumping 150 gallons per minute was obtained and positioned at the edge of the river about 300 meters from the dambo. We were able to flood a 19,000 ft^2 area and maintain flood levels during an 18 day period by pumping approximately 3 million gallons of water. The flooding induced the hatch of many millions of Aedes eggs in the dambo. Pupae were collected and returned to the laboratory so that Rift Valley Fever (RVF) virus isolation attempts could be made with the emerging adults. About 5000 reared adult *Ae. lineatopennis* in 101 pools were tested for RVF and five isolations of RVF were made. In reared adult males the isolation rate was 1 in 1600 and in adult females it was 1 862. Four of the five isolations killed hamsters on the original inoculation. Reisolation attempts with the original mosquito suspension failed.

This data suggests that transovarial transmission of RVF virus may occur in ground pool breeding *Aedes*. This observation, together with epidemiological and ecological association of these species with RVF epizootics supports the hypothesis that RVF virus is maintained, at least in part, during the interepizootic periods by transovarial transmission in *Aedes* mosquitoes.

A Blood Meal Analysis of Engorged Mosquitoes Found in Rift Valley Fever Epizootic Areas

This study examined 800 blood fed mosquitoes trapped during and following a period of particularly heavy rainfall (October-December 1982) by an enzyme immune assay system. The most common mosquito species encountered over the trapping period was *Aedes lineatopennis* (Ludlow), which comprised more than half the blood fed specimens examined. Of the 389 specimens shown to contain haemoglobin 333 (85.6%) had taken bovine blood meals. Three other common *Aedes* species, *Ae. dentatus* (Theobald), *Ae. cumminsii* (Theobald), and *Ae. sudanensis* (Theobald) were 90%, 79.1% and 92.3% respectively, positive to the specific antibovine conjugate. Comparatively few *Culex* specimens were obtained, but of these 93.3% of the haemoglobin positive *Culex antennatus* (Becker) had taken bovine blood meals. One specimen of *Ae. linentopennis* had taken a human and one a rabbit blood meal. A single *Ae. dentatus* had fed upon a giraffe and one *Ae. cumminsii* upon a dog or jackal and 2 on a rabbit. The anti-rabbit reactions were weak, suggesting that the blood was originating from some non-rabbit lagomorph. Four specimens of *Ae. circumluteolus* (Theobald) and one *Ae. quasiunivittatus* (Theobald) had fed upon a horse. Of the 104 specimens shown to contain haemoglobin but not identified by the 13 specific conjugates, 8 were found to react strongly when tested later with the broad spectrum anti-bovidae conjugate. These 8 must have fed on some other less common species of wild ruminant, most likely impala (*Aepyceros melampus*) or kongoni (*Alcelphus buselaphus cokii*). This leaves, however, a further 96 (13%) blood fed mosquitoes that could not be identified.

Rainfall and Epizootic Rift Valley Fever

An analysis of rainfall at five different sites in Kenya where epizootics of RVF occur has been undertaken from 1950 to the present. The correlation between the RVF epizootics and the rainfall has been documented. The epizootics of 1951-1953, 1961-1963, 1967-1968 and 1977-79 are clearly and consistently associated with periods of high positive surplus rainfall. Some few clinical cases were seen later in 1975 and 1982, correlating with the lower positive values of the statistic at these times. No virus activity has been recorded during periods of negative surplus rainfall. The key factors in the association would appear to be the widespread nature of the rainfall over several sites to give greater than the annual mean, together with a level of persistence of the rainfall measured by the frequency of rain and its continuance over a long period of time. The apparent failure of heavy local rain to produce limited epizootics suggests that some other factors are involved, in addition to the rainfall.

Mosquito Species Encountered in A Flooded Grassland Dambo in Kenya

The larvae and pupae of mosquitoes found in a flooded dambo on a bushed grassland in Central Province, Kenya were monitored during the short rainy season. The densities of the immature stages of 8 species were recorded daily for a one month period. *Aedes cumminsii mediopunctatus*, *Ae. lineatopennis* and *Ae. sudanensis* were collected for 8, 9 and 18 days respectively and each disappeared after one generation. *Aedes lineatopennis* and the *Culex* spp. specimens were collected in much greater densities than in a forest dambo studied previously. The 3 *Aedes* spp. described in this study were found in very large numbers only in association with flooded dambo situations. The only ecological change that increases the surface area of the standing water in this area and produces the conditions necessary for large numbers of mosquitoes to be produced is the flooding of the dambos.

Aerial Collection of Culicoides schultzei (Diptera:Ceratopogonidae) in Kenya

The spread of bluetongue and ephemeral fever viruses, within and outside endemic areas in East Africa, is thought to be caused by the movement of infected *Culicoides* which become airborne during the passage of the Intertropical Convergence Zone (ITCZ). However, there has been no direct evidence of *Culicoides* involvement in the ITCZ air movements. The ITCZ is an equatorial belt of low barometric pressure where, at low levels, air flowing from the northern and southern hemispheres converge. Collections were attempted by making 3 flights (Jan. 7, 9, 12, 1984) in a Cessna 150 over a 200 km^2 section of bushed grassland (1°S, 37°E; 1500 m) in ecological zone III.

Twelve specimens of the *Culicoides schultzei* group (sensu Khamama and Kettle 1971), were collected on Jan 9 at 1950 m. The collection was comprised of 9 males and 3 females. A precise identification was not possible due to the damaged condition of the specimens. No specimens were collected on Jan 7 or 12. The total volume of air sampled during each collection flight was approximately 580 m^3. The mean density of insects sampled was one insect in 48.3 m^3. The collection of a vector of bluetongue and ephemeral fever virus at 450 m above ground level lends support to the theory that airborne wind carriage of insects is a means of disease dissemination.

The Sudan Dioch (*Quelea quelea aethiopica*) and Rift Valley Fever

Dr. R. A. Alexander reported in 1957 that the seasonal migrations of the Sudan dioch (*Quelea quelea*) were responsible for introduction of RVF into different parts of the Rift Valley in Africa. An experiment was carried out to determine whether the Sudan dioch was susceptible to RVF.

A strain of RVF isolated from *Aedes lineatopennis* in 1978, (Davies and Highton 1980) was used to inoculate the birds. This had been passaged 3 times in infant mouse brain and once in Vero cells. Thirty-six birds were inoculated with $10^{5.5}$ $RCID_{50}$ in 0.1 ml volumes into the subcutaneous tissue and muscle of the pectoral area. These birds were killed at 12 h intervals thereafter for five days. Blood and liver samples were taken from each bird for virus isolation attempts. Two golden Syrian hamsters (*Mesocricetus auratus*) were inoculated intraperitoneally with 10^{-1} dilutions of blood and two with 10% suspensions of liver homogenate in a transport medium. No isolations of RVF virus were made from any sample. No antibody to RVF was detected in 3 birds which had received RVF virus, although there was a slight reduction of the cytopathic effects at 1/10 dilutions of their sera, compared with uninoculated control birds sera. A control RVF sheep immune serum had a titer of 1/1280.

Predation On Emerging Adult Mosquitoes By *Brachvdeutera munroi* (Diptera: Ephydriade)

The larval stages of *Brachydeutera munroi* Cresson were observed to attack quiescent mosquito pupae and kill the emerging adults. We observed that all stages of the fly larvae were predatory on *Aedes cumminsii* (Theobald) and *Ae. lineatopennis* (Ludlow). We did not observe any predation on *Ae. sudanensis*, although the numbers examined were very small (10). The fly larvae attacked pupae resting at the surface at the time of imaginal ecdysis. The larva penetrated the pupal cuticle along the ecdysial line as it opened along the middorsal surface of the thorax. As the body of the adult mosquito emerged from the pupal cuticle the predatory larvae were observed to attack near the intersegmental membranes of the abdomen and killed the emerging adults after only partially shedding the pupal cuticle. The head and thorax remained intact in all mosquitoes observed. The fly larvae were not observed to prey on mosquito larvae. Pupae that had not started to molt would swim away from the fly larva when contacted and not be harmed. All attacks on mosquito pupae were made by a single fly larva. These observation indicate that *Br. munroi* may act as an important predator of emerging adult mosquitoes.

PROGRESS IN RABIES RESEARCH

A case of rabies was diagnosed in an American Peace Corps Volunteer (PCV) who had received a full pre-exposure series of intradermal (ID) human diploid cell rabies vaccine (HDCV) before exposure to a rabid dog. This case prompted investigations of the ID use of HDCV. We have found that persons immunized in developing countries have unexpectedly low anti-rabies antibody titers after the pre-exposure series. Groups of volunteers vaccinated in the United States developed higher titers than native Kenyans, and Kenyans developed higher titers than PCV's vaccinated in developing countries. Currently, there is no explanation for the poor responses to HDCV in Kenyans or PCV's, although both chloroquine and other concurrent immunizations have been shown to supress the antibody response to HDCV in controlled trials in the U.S.

PROGRESS IN HUMAN T-CELL LEUKEMIA VIRUS-3 (HTLV-3) RESEARCH

Twenty-one percent of Kenyan subjects have positive reactions in an ELISA assay for antibodies to HTLV-3. The percentage of positive reactions increased with age and demonstrated dramatic regional differences. Among the Turkana, 50% of the subjects were positive, while only 6% of the Masai and 11% of the urban residents of Nairobi were positive. Patients with visceral leishmaniasis had similar rates of positivity as the general population (14%) however patients with undiagnosed splenomegaly or schistosomiasis had very high rates of positivity in the small sample tested.

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b. CONTRACT/GRANT NUMBER		84	8.0	820
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23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Research efforts in this department continue to be directed toward gastrointestinal diseases of military importance. Focus is on enteropathogenic bacterial diarrheal disease caused by pathogenic E. coli, but also Shigella and Cholera. These have critical military relevance because of their influence on troop mobility, particularly following deployment of units to new areas.

24. (U) Studies of bacterial diarrhea are being conducted in 4 general areas: 1) mucosal adherence as a determinant of bacterial colonization; 2) intestinal immune response to bacterial infection; 3) pharmacologic modification of effects of infections on intestinal transport, and 4) motility. Studies utilized preparations of intestinal membrane fractions, bacterial adherence factors (pili), isolated and functionally characterized intestinal mononuclear cells, and in vivo acute and chronic recording of intestinal myoelectric activity.

310-8409 Mucosal Adherence: Structure of colonization factor antigen/II (CFA/II) has been further defined and additional lots of CFA/II have been prepared for further testing for mucosal immunogenicity. Immunology: Peyer's patch lymphocytes were immunized in vitro and immunoglobulin production enhanced. Peptide with specific antigenic site, binding site, and adjuvant was synthesized. Transport: Results from vesicle studies suggest that cholera toxin stimulates D-glucose absorption by increasing glucose-Na^+ coupling coefficient across the brush border membrane. Motility: Abnormal propulsive ileal and colonic motor responses develop during enteropathogenic E. coli infection and correlate better with mucosal adherence than with luminal colonization. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

Project: 3M162770A870 MEDICAL DEFENSE AGAINST INFECTIOUS DISEASE

Work Unit 051 Gastrointestinal Diseases of Military Importance

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PROBLEMS AND OBJECTIVES

i. Role of Mucosal Adherence in Bacterial Colonization

Colonization of the small intestine is a prerequisite for the production of clinical diarrhea by many enteropathogens, including the enteropathogenic (EPEC) and enterotoxigenic (ETEC) groups of E.coli. The latter group (ETEC) is responsible for a majority of cases of Traveler's diarrhea. One important mechanism promoting small bowel colonization is the adherence of bacteria to the intestinal mucosal surface. In order to develop effective means of preventing and treating bacterial diarrhea we have been attempting to answer the following questions: What are the structures (adhesins) on the surface of bacteria which enable them to specifically attach to the host's mucosal cells? What are the receptors, or binding sites, for bacteria on the host's intestinal cells? What immunologic or pharmacologic means can be used to prevent or reverse the adherence of pathogenic bacteria to the intestine? Can an adherence antigen be used as an effective oral vaccine against enteric infection with pathogenic E.coli?

ii. Role of Host Immune Mechanisms

The mechanisms of local immunization and the regulation of local immune responses at the level of the intestinal mucosa are being studied to develop better oral vaccines. Synthetic peptide immunogens are being developed as oral vaccines to give protective intestinal mucosal immunity. The goal is to have vaccines giving long term T-lymphocyte immunity without interfering secretory antibody production, thus allowing subsequent booster oral

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immunization for both specific T-lymphocyte and local secretory antibody immunity. Peptide antigenic site modification is the approach being taken leading towards cross-reactive T-lynphocyte immunization without cross-reactive antibody formation as a prototype for initial intestinal immunization allowing for booster immunization with the modified peptide antigen. Lymphocytes from the gut associated lymphoid tissue (GALT) are being used for *in vitro* immunization and challenge with synthetic peptide antigens.

iii. Alterations of Intestinal Transport

The objective is to obtain a better understanding of functional properties of the intestine. We are particularly interested in the secretion of fluid and ions into the intestinal lumen. We hope to obtain further information on the nature of the secretory process (processes), including anatomical location, mechanism of operation and its sensitivity to various reagents, both stimulatory and inhibitory. These secretory processes are of particular interest with respect to bacterial diarrhea because they may be involved in the relevant pathophysiology. Attempts to answer the following questions may aid the development of more effective or simpler therapies for bacterial diarrhea. What are the mechanisms for salt and water transport under normal conditions and in the secretory state induced by bacterial toxins and other secretory stimuli? Can pharmacologic agents reverse the salt and water secretion induced by bacterial toxins and other secretogogues?

iv. Alterations of Intestinal Motility

Disabling, sometimes lethal, gastrointestinal manifestations of enteric infection include anorexia, nausea, vomiting, abdominal pain and cramps, diarrhea and (paradoxically) constipation , and bacterial colonization and overgrowth. These derangements suggest disruption of gastrointestinal motor function and transit. The immmediate objective of this section is to describe the effects of enteric infection on gastrointestinal motility and transit and to relate these changes to symptamatology, pathophysiology, current treatment modalities, and resolution of infection. Mechanisms and neurohumoral pathways for these effects and their complex cause/effect relationships with the normal enteric flora and infectious agents will be assessed. The goal will be to apply this information to the development of more rational and effective prophylaxis and therapy of diarrheal diseases.

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PROGRESS

1. Role of Mucosal Adherence in BActerial Colonization

Traveler's diarrhea (TD) is of great military concern because of the high likelihood that it will have a debilitating effect on troops newly deployed to endemic areas. TD is most commonly caused by ETEC strains which produce surface pili (or fimbriae) which function as colonization factor antigens (CFAs). Two antigenic types (CFA/I and CFA/II) were initially recognized in human ETEC isolates, but additional CFAs have now been recognized and more undoubtedly remain to be discovered. Studies in analagous animal infections show that susceptible hosts can be protected from ETEC colonization by passive administration of antipilus antibody. In theory, an effective vaccine against ETEC-induced TD should stimulate a local secretion of IgA against relevant CFAs and oral adminstration of CFAs should induce a maximal response.

We have previously shown that preparations of CFAs from ETEC can be sterilized either by radiation or formalin and yet retain their mucosal immunogenicity in animal systems. In collaboration with Dr. S. Berman at the Forest Glen facility we prepared an initial lot of radiation-sterilized CFA/II pilus antigen which was sterile, immunogenic in animals and safe for oral administration. An IND was obtained to permit testing of this vaccine product in human volunteers under the supervision of Dr. M.M. Levine and colleagues at the center for Vaccine Development, University of Maryland. This CFA/II preparation from the O6:H16 *E.coli* stain M424C1 was tested in 1C healthy volunteers who each received eight 1.7 mg oral doses after cimetidine and with bicarbonate. No adverse reactions were noted. Only 2 of the volunteers developed significant serum IgG or secretory IgA responses to the vaccine preparation. Eight voluunteers were challenged with an infective dose of a toxin positive, CFA/II positive ETEC. 6 of 8 controls developed diarrhea as opposed to 3 of 8 vaccinees. Only one of the two volunteers who developed an immune response following vaccination was challenged and this volunteer did not develop diarrhea. The results suggest that although immunization with CFA/II might be protective, the orally administered CFA/II preparation was poorly immunogenic. This was especially true in comparison with the protection achieved by administration of a naturally occuring, CFA/II positive, toxin negative strain.

Additional information was obtained on the characterization of the components of the CFA/II vaccine. "CFA/II" is not a single antigen, but composed a fine fibrillar antigen termed CS 3 which is common to all CFA/II strains, and variable pilus antigens which have been termed CS2 and CS3. Our vaccine contined predominately CS3 (95%) and lesser amounts of CS1. In the vaccine studies, only one vaccine developed antibody rises to CS3 and none developed rises to CS1.

An additional lot of the radiation sterilized CFA/II vaccine was prepared, as well as a lot of formalin sterilized CFA/II, by Dr. S. Berman. In addition, lots of formalin killed preparations of whole ETEC organisms bearing CFA/I or CFA/II have been prepared for future testing.

Because of the difficulties in carrying out multiple trials of immunization and challenge in human subjects, a major focus of the department has been the definition of the determinants of enteroadherence in a naturally occurring diarrheal disease of rabbits, which can serve as a model for human intestinal infection. This rabbit infection with the O15:K-:NM E.coli strain RDEC-1 has emerged as a model highly analogous to human disease caused by the enteropathogenic E.coli (EPEC) serotypes. We have developed evidence that the enteroadherence of RDEC-1 to rabbit intestine is conferred by the expression of specific pili which we have designated AF/R1 since they are the first pilus attachment factor described with specificity for rabbit intestine. These AF/R1 pili can be isolated in quantity from large scale batch cultures of the organisms and are routinely utilized in studies of mucosal immunization in the rabbit model. Per oral inoculation of rabbits with AF/R1 pili has limited intestinal colonization following subsequent challenge with infective doses of the organisms.

AF/R1 pilus expresion by RDEC-1 is determined in part by growth conditions. Studies have been conducted to determine the nutritional requirements and optimal physical conditions for AF/R1 pilus expression in order to optimize pilus production for harvest as well as to better understand conditions in the intestinal lumen which might promote expression of this virulence determinant.

Expression of AF/R1 pili during the course of RDEC-1 infection has been demonstrated by following the development of a specific secretory IgA response to this antigen follwing oral infection utilizing animals with the isolated Thiry-Vella loop. In these studies, antibody response to type 1 (or common) fimbriae could not be demonstrated during the course of RDEC-1 infection. Although the role of host-specific, non-mannose sensitive pili such as AF/R1 in enteric E.coli infection is well established, a similar role for type 1 mannose sensitive adhesions is controversial. To determine if E.coli expressing type 1 pili differ in affinity for intestinal membranes and other surface properties, we grew a series of E.coli strains in static broth to promote type 1 and suppress NMS pilus expression. Results indicated that E.coli expressing type 1 pili vary in their ability to adhere to intestinal membranes and that this adherence correlates better with hydrophobicity than with hemagglutination profile. These studies suggest that type 1 pili are functionally heterogeneous and that some type 1 pili may play a role in *in vivo* enteroadherence.

Interactions of piliated *E.coli* with intestinal membranes have been well studied; however pilus-mediated interactions with intestinal mucus are not well defined. Luminal mucus might provide a site for initial intestinal colonization and a source of nutrients for enteric pathogens. *In vivo* studies of RDEC-1 infection of rabbits demonstrated interactions of the organisms with the mucus. *In vitro* studies of interactions of nonpiliated, and type 1 and AF/R1-piliated RDEC-1 with purified mucus glycoprotein demonstrated that piliated, but not non-pilated, organisms adhere to intestinal glycoproteins derived from both microvillus membranes and the luminal mucus. They support the concept that the mucus layer could serve as a site for bacterial replication prior to enteroadherence.

ii. Role of Host Immune Mechanisms

In order to better understand the intestinal immune response leading to antibody production, the isolated Peyer's patch mononuclear cells were studied. The culture conditions and cellular requirements for pokeweed mitogen (PWM) induced polyclonal B-lymphocyte activation of rabbit Peyer's patch and spleen cells was determined. Although monocytes/macrophages did not appear to be critical, the immunoglobulin responses to PWM were T-lymphocyte (T-cell) dependent.

Using PWM as a control, the mode of action of a newly described poyclonal B-cell activator factor found in rabbit breast milk cell supernatant was studied. The factor stimulated both B-cell enriched (T-cell depleted) and monocyte/macrophage depleted lymphocyte populations from Peyer's patch and spleen indicating the lack of both T-cell and monocyte macrophage requirements for polyclonal B-cell activation. The polyclonal B-cell activator does not require blast transformation and is not mitogenic for either Peyer's patch or splenic lymphocytes. A 66 kilodalton (KD) protein in the milk cell supernatant was found to contain the B-cell activation property. Although the cell of origin is yet to be determined, this polyclonal B-cell activator protein can be further characterized and used in both *in vitro* and *in vivo* experiments leading towards enhancement of secretory antibody response in the intestine.

The approach used in characterizing the polyclonal B-cell activator factor was also used to determine the culture conditions and cellular requirements for antigen-induced B-cell activation of the rabbit Peyer's patch and spleen. The AF/R1 pilus protein of the EPEC strain RDEC-1 was used as the antigen. The optimal conditions for both primary and secondary in vitro immunization were similar except for culture duration. Both optimal cell density and antigen dose were determined. T-cells and monocyte/macrophages were required for protein antigen-induced antibody production for both primary and secondary responses. Of note was the inability to induce a primary antibody response in the spleen cell cultures. This in vitro system may be very useful in the development of synthetic peptide antigens for use as immunogens in synthetic oral vaccines against E.coli induced enteric disease.

To enhance the effective immunogenicity of synthetic peptide antigens, a synthetic peptide immunogen to include a binding site for epithelial cells to enhance absorption and an immunoadjuvant to enhance immunogenicity is being developed as a prototype approach for synthetic peptide vaccine development. The GM_1 ganglioside-binding 21 AA peptide fragment of cholera toxin B chain has been synthesized as well as extended synthesis to include the 8AA peptide (GLY-ASN-THR-ILE-VAL-ALA-VAL-GLU), an antigen site for rabbit lymphocytes. A collaboration with Dr. Louis Chedid, Pasteur Institute, Paris, France, has been developed whereby he will conjugate derivatives of muramyl dipeptide onto the 8AA, 21AA and 29AA peptides. These conjugates will then be tested for their ability to maintain antigenicity by binding to monoclonal antibodies and/or binding to GM_1 ganglioside. If antigenicity and GM_1 binding are maintained, these conjugates will be tested for their ability to enhance in vitro immune responses.

In order to immunize isolated rabbit Peyer's patch and ileal lamina propria lymphocytes with a small synthetic peptide immunogen, a 13 AA peptide containing the 8AA in the N-terminal portion (a known rabbit immunogen) was synthesized and is currently being purified and chemically analyzed. Also a series of peptides 7-13, 6-13, 5-13, 4-13, 3-13, 2-13 were obtained during the 13AA peptide synthesis. These peptides will be used to determine the epitopes in the 8AA peptide that a series of monoclonal antibodies which bind to the 8AA peptide recognize. These monoclonal antibodies were obtained in collaboration with Dr. Kaprowski, Wistar Institute, Philadelphia, PA. The epitope specificity of these monoclonal antibodies will make them critically useful in the development of peptide analogs which have lost antibody binding but maintain T-cell recognition.

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iii. Alterations of Intestinal Transport

The rates of D-glucose uptake by the mucosal brush border membrane vesicles prepared from normal and cholera toxin-treated rat small intestine were studied. Vesicles pre-treated with valinomycin, in order to eliminate the electrical potential difference between the interior and the exterior, were studied along with the vesicles not treated with valinomycin. Valinomycin increased the rate of D-glucose uptake when the vesicles were bathed in NaCl and Na isethionate solutions whereas valinomycin decreased this rate in vesicles bathed in NaSCN. Thus the order of permability of the brush border membrane is SCN, $Na^+ > CL =$ isethionate. In the presence of valinomycin, cholera toxin treatment still significantly increased the rate of D-glucose uptake suggesting that the increased D-glucose absorption in the vesicles, as well as that observed in the Ussing chambers, is due to an increase in the coupling ratio of D-glucose to Na in the carrier mediated transport process across the brush border membrane. These results have ruled out the other possibility that the cholera toxin-stimulated increase in D-glucose absorption resulted from an increase in the electrochemical potential difference of $Na+$ across the brush border membrane. In addition, development of the technique for solute uptake measurement in basolateral membrane vesicles is underway.

Progress has been made in the development of standard techniques for quantitative assessment of intestinal transport in human volunteers and patients. Using a triple-lumen intestinal perfusion tube, 37 separate studies of sequential jejunal and ileal transport have been performed on twelve volunteers and four patients with chronic diarrhea.

Studies performed in normal volunteers have established normal ranges for water, sodium, potassium and chloride transport. In addition, eight volunteers have been placed on a 17 meq sodium diet for three days in a metabolic unit to assess the effect of sodium depletion on ileal sodium and water transport. Preliminary analysis of the first three studies has demonstrated the efficacy of salt depletion in that there have been significant increases in volunteer weights and plasma renin levels. Changes in ileal transport of water and electrolytes were noted with a trend toward ileal secretion of potassium associated with salt depletion. This suggests the presence of a renin/aldosterone effect in the ileum. This has not previously been demonstrated in humans.

One patient studied was a 54 year old man with extremely high serum vasoactive intestinal peptide (VIP) levels but without the clinical syndrome of pancreatic cholera. Perfusion studies performed prior to and during the infusion of porcine VIP

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demonstrated that the patient was secreting a biologically inactive form of the hormone.

iv. Alterations of Intestinal Motility

We have previously reported that migrating (MAPC) and stationary (SAPC) action potential complexes are abnormal peristaltic and non-peristaltic intestinal motor responses to a variety of sensory stimuli that occur in the setting of diarrheal enteric infection: enterotoxins, dietary lectins, mocosal damage and luminal distention. Using the same anesthetized *in vivo* rabbit model, we studied these motor responses during a naturally occurring EPEC infection with *E. coli* strain RDEC-1. Both MAPCs and SAPCs developed in the ileum and colon during infection. The magnitude of motility change correlated better with bacterial mucosal adherence than with luminal colony counts. We conclude that diarrheal motility responses are a primary event in enteroadherent disease. In addition, colonic slow wave frequency (non-contractile pacemaker potential) accelerated during infection, suggesting that enteroadherence may either act by increasing pacemeker frequency or by facilitating excitation-contraction coupling at a given pacemaker rate. Computer programs to allow more precise and complete analysis of these events are under development.

Preliminary investigation of response pathways in this model suggest that cholinergic agonists produce, muscarinic blockers inhibit, and the myenteric plexus tonically inhibits, these motor patterns. Using fetal rat intestine allotransplanted to the back of another rat, we have shown that MAPCs and SAPCs are intrinsic to intestinal smooth muscle and its myenteric plexus, and that suppression and coordination of these responses requires, in part, external innervation. These findings suggest that peripheral control mechanisms are of major importance in diarrheal motility responses and that these mechanisms should be susceptible to pharmacologic manipulaton with minimal systemic consequences.

The control mechanisms of feeding on gastrointestinal motility was investigated in unanesthetized, chair-adapted monkeys utilizing newly developed computer programs to accurately quantitate the increased spike response to feeding. Carbohydrate ingestion and serum glucose concentrations correlated with disruption of fasted motility pattern (migrating motility complex) and the initiation and duration of postprandial spiking while fat ingestion did not. By suggesting that different dietary constitutents have significant and differing effects on gastrointestinal motility, important questions about dietary therapy and aggravation of disorders with abnormal motility (in particular enteric infections) have been raised.

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FUTURE PLANS AND RECOMMENDATIONS

i. Role of Mucosal Adherence in Bacterial Colonization

Studies of the use of purified *E.coli* adherence antigens as mucosal immunogens to provide protection against enteric infections will continue both in animals and in human studies. In the animal model, using AF/Rl pili from RDEC-1, experiments will be continued to determine the optimum timing, dose and route of administration of pilus antigen to induce a local secretory immunoglobulin response. This approach will be expedited using the rabbit Thiry-Vella loop model, since recent results indicate that antigen secretion in the isolated loop is representatvie of antibody secretion in intact intestine during the course of RDEC-1 infection. Other methods for quantitating sequential secretory antibody responses in intact animals will be explored including the use of intestinal wash out techniques.

Additional studies with new lots of CFA/II CSl/CS3) will be undertaken to determine whether the limited immunogenicty of the preparation was the result of failure of delivery of the antigen preparation to the mucosal immune system (i.e the specialized Peyer's path epithlial M cells). Failure of antigen delivery may have been due to alteration of the antigen structure by gastric acid, or more likely, by pancreatic proteases. Alternatively, the antigen may have been taken up by the absorptive epithelium for which it has demonstrated affinity *in vitro*. Since the antigen may have been adversely affected by the methods of gamma radiation used for sterilization, experiments will be performed to measure local immune response of the antigen following direct instillation into the intestine by intestinal tube (thereby bypassing stomach and pancreas). *In vitro* studies will be undertaken to determine the influence of pH changes and proteases on the antigenicity of the preparation using ELISA. Effect of antigen form on the immune response will be investigated by examining the immune responses to orally administered, formalin-killed organisms.

The rabbit model of RDEC-1 infection will be used to explore these possibilities since it provides a model for pilus (fimbria) mediated enteroadherence. Studies will be undertaken to determine whether pili with high affinity for host intestinal cells (i.e. AF/Rl in the rabbit) have greater or less immunogenicity than those with low affinity for host mucosal cells (i.e. CFA/II in the rabbit). These studies will explore the question of whether binding of adherence antigen to the mucosal cells inhibits antigen exposure to the mucosal immune system.

This department will begin to utilize the techniques of molecular genetics to examination the relationships among enteroadherence, colonization and immune protection. Studies of mucosal immunization against CFA/II have indicated that live organisms expressing adherence antigens provide superior immune protection to that provided by killed antigens. Ultimately, avirulent, but transiently colonizing engineered strains expressing adherence antigens may be the most effective vaccines. Initial studies will be performed to identify and clone the plasmid associated genes responsible for AF/R1 pilus expression in RDEC-1. Expression of AF/R1 pili in avirulent strains should provide both prototype vaccine candidates for testing in the animal model, as well as more efficient means of pilus production (multiple copy plasmids). Attempts will also be made to cure RDEC-1 of its virulence plasmid in order to further explore the relations between pilus expression, immunity and virulence. In future years, principles determined in these basic studies with RDEC-1 will be appled to human pathogens for vaccine development.

Recent demonstration that type 1 pili on RDEC-1 confer *in vitro* mucosal adherence to enterocyte membranes, and that type 1 pili from different *E.coli* strains have considerable functional heterogeneity with regard to their enteroadherence, reopens the question of the role of type 1 pili in intestinal colonization. Immune protection by type 1 pili prepared from RDEC-1 will be examined.

Monoclonal antibody reactive with AF/R1 pili has been developed by the Department of Bacterial Diseases. These antibodies will have multiple application including purification of AF/R1 components for large scale production of pure pilus antigens, evaluation of immune relations of AF/R1 to other adherence antigens, quantitation of pilus expression, and identification of genetically engineered strains producing this product.

ii. Role of Host Immune Mechanisms

Rabbit enteropathogenic *E.coli* strain RDEC-1 contains a specific pilus protein attachment factor (AF/R1). Orogastric immunization with isolated AF/R1 pilus protein limits intestinal colonization following challenge with the virulent organisms. If the pilus protein contains a free N-terminal amino acid, sequential AA analysis will be attempted. The N-terminal peptide region will be synthesized by extending the synthesis of a 21AA peptide representing the GM_1 ganglioside-binding site of the beta chain of cholera toxin. A synthetic immunological adjuvant derivative of muramyl-dipeptide (MDP) will be conjugated onto the synthetic polypeptide. The resulting synthetic immunogen containing a peptide antigenic site (AF/R1 pilus protein): 21 AA absorptive carrier (binds to GM_1 ganglioside): adjuvant (MDP derivative) will be studied for its ability following orogastric immunization to limit intestinal colonization and subsequent enteric disease caused by RDEC-1.

The synthetic peptide (GLY-ASN-THR-ILE-VAL-ALA-VAL-GLU) is an antigenic site for rabbit ileal lamina propria T-lymphocytes and contains a smaller antigenic site for antibody binding. The larger peptide antigenic site for the T-lymphocyte suggests that conformation of the antigenic site may be more important for the T-lymphocyte antigen receptor than for antibody binding. Since the conformation of the octapeptide is now known, the development of two analog series may be possible with the use of the already established computer-assisted molecular modelling utilizing energy minimization strategies. One series would have basically different primary structures and the other series would have very dissimilar conformations with minimal changes in primary structure. These analogs would be synthesized using the already established peptide synthesis capability. The peptide conformation would actually be determined using the 600 or 750 MHz high resolution proton NMR facility at Carnegie-Mellon University. The analog peptides would be tested for the presence or lack of cross antigenicity with the original octapeptide using rabbit ileal lamina propria T-lymphocytes in vitro and antibody binding. This study will be testing the hypothesis that the conformation of a peptide antigenic site is more critical than the amino acid sequence for recognition by sensitized T-lymphocytes in contrast to antibody binding where the opposite is true. Several peptide analogs may be found which give good T-lymphocyte cross reactivity but no cross-reactive antibody binding. One of these peptide analogs would then be constructed into a synthetic vaccine consisting of the peptide analog: 21AA peptide absorptive carrier: binds to GM_1 ganglioside): adjuvant (MDP derivative). This conjugate will then be studied for its ability to enhance intestinal immunity following orogastric administration such that booster orogastric immunization can take place with the unmodified 8AA peptide conjugate giving long-standing T-cell immunity and secretory IgA immunity.

iii. Alterations of Intestinal Transport

We will concentrate on studying the electrical properties of, and solute transport processes across, the individual cell membranes of the intestinal epithelium. In the past year we established the technique for measuring the rate of D-glucose

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uptake by brush border membrane vesicles. In FY 85 we will develop the technique for purifying baso-lateral membrane vesicles to study solute permeability across that membrane. The baso-lateral membrane vesicles will be calibrated by measuring amino acid uptake rate and marker enzyme activities. Then the effect of magnesium concentration on sodium transport will be studied in both brush border and basolateral membrane vesicle preparations in order to reconcile the results obtained from Ussing chambers.

Information on the electrical resistance and capacitance of the apical and basolateral cell membranes as well as of the intracellular shunt pathway is of particular importance for better understanding of how the barriers (individual epithelial cell membranes) contribute to the overall properties of the intestine. Such information can be obtained by a current pulse method and mathematical modelling analysis. A current is applied across the intestinal mucosa *in vitro* and the response in transmural and/or transmembrane electrical voltage in microsecond range are fitted to an epithelial cell model. A least squares curve fitting and mathematical analysis produce estimates of the desired membrane electrical properties. In FY 85, we will acquire the instruments including a waveform digitizer and perform experiments to obtain baseline information on the membrane electrical resistances and capacitances.

Studies to be initiated in FY 85 will assess the efficacy of substituting various sugars in the standard World Health Organization oral rehydration formula as regards improving intestinal absorption of water and sodium. The current formula, which contains glucose, is more expensive and less easily prepared in a non-laboratory setting than solutions containing sucrose or rice flour as the source of carbohydrate. Dissacharides or carbohyrate polymers may further increase sodium and water absorption by providing more substrate for glucose-sodium cotransport without increasing the osmolality of the rehydration formula.

The triple-lumen technique will also be utilized to assess quantitatively the intestinal antibody response to immunization with purified *E.coli* pili. The ability to ascertain quantitative immunoglobulin response by marker perfusion technique will better allow determination of the most appropriate dose and delivery site for *E.coli* antigen.

iv. Alterations of Intestinal Motility

Studies on the motor effects of enteric infection and manipulation of neurohumoral pathways are continuing in

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anesthetized animals. These observations are being extended and combined in unrestrained, unsedated animals with chronically implanted electrodes. Specifically, the roles of potentially therapeutic agents (metoclopramide, cisapride, pyridostigmine), antimotility drugs (loperamide, opiates, scopolamine), and diet will be examined in the development, severity and resolution of enteric infection using implanted electrodes, strain gauges, and C^{14} PEG transit times to simultaneously evaluate electrical, mechanical and propulsive events. The relationships among diet, motility and endogenous gut flora will be investigated using a newly described rat model of lectin-induced bacterial overgrowth. Long term projections are to extend these studies to primates and humans (using intraluminal strain gauges and electrodes) in order to directly study the pathogenesis and therapy of significant human enteric infections.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA 305976	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A870	AI	052 WWSJ
b. CONTRIBUTING				
c. XXXXXXXXX	STOG 82/83-6.2/3			

11. TITLE *(Precede with Security Classification Code)*
(U) Exploratory Vaccine Development Against Leishmaniasis

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE — EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE — d. AMOUNT
e. KIND OF AWARD — f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	0.0	00
85	2.0	68

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Div of CD&I
b. ADDRESS *(include zip code)* Washington, DC 20307-5100	b. ADDRESS Walter Reed Army Institute of Research Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Hockmeyer, W T
d. TELEPHONE NUMBER *(include area code)* 202-576-3551	d. TELEPHONE NUMBER *(include area code)* (202) 576-3544
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Ballou, W R g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Vaccine; (U) Antigens; (U) Protozoa; (U) Leishmania; (U) RAMI

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23 (U) The objective is to produce a safe, efficacious promastigote vaccine against Leishmania donovani which poses a significant threat to military operations in endemic areas.

24 (U) The approach used is to clone the gene encoding for several candidate promastigote surface antigens and to produce these antigens in vitro for immunogenicity testing.

25 (U) None.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 0B 6489
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A870	AT	072 WWK2
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 82/83-6.2/3			

11. TITLE (Precede with Security Classification Code)
(U) Assessment of Infectious Diseases of Military Importance

12. SUBJECT AREAS
0605 Clinical Medicine 0606 Environmental Biology

13. START DATE: 72 07
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-house

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	3.0	121
85	3.0	165

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER (include area code)
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS
Division of Preventive Medicine
Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
Miller, R N
d. TELEPHONE NUMBER (include area code)
(202)-576-3553
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
Takafuji, E T
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
Brundage, J

21. GENERAL USE FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Epidemiology; (U) Infectious Disease; (U) Risk Assessment; (U) Data Bases (U) Volunteers; (U) ~~RAD I~~; [illegible]

23 TECHNICAL OBJECTIVE 24 APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To identify, define, and evaluate known and potential causes of disability in military populations using appropriate epidemiologic techniques. To develop epidemiologic methods in the assessment of militarily relevant infectious disease risks. To apply this information to the prevention and control of diseases in military populations in order to maintain combat effectiveness and readiness.

24. (U) Contemporary epidemiologic methods are applied to causes of disability in military populations. Multidisciplinary approaches are utilized and new methods developed as indicated. Military deployments to high risk areas are monitored for infectious disease problems and infectious disease threats are defined.

25. (U) 8310-8409 Medical surveillance of military deployments include monitoring of troops training in Panama, Honduras, and the Middle East and review of current worldwide disease occurrence information, in order to identify current disease threats for military forces. Medical surveillance of selected units on missions to geographical areas where infectious disease risks are high, such as Central America and Southwest Asia, often require pre- and post-deployment evaluations. Incidence of acute respiratory disease at basic training posts are being monitored, and studies are programmed to determine etiologies of ARD admissions. Specific disease problems such as intestinal helminthiasis in the tropics, streptococcal disease in recruits, hepatitis A and B in prisons and in overseas areas such as Korea, Korean hemorrhagic fever in U.S. forces, and leptospirosis associated with training in the tropics are issues being currently addressed. Health threat assessments are being developed based on specific scenarios and geographical areas. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

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Project 3M162770A870 MEDICAL DEFENSE AGAINST INFECTIOUS DISEASE

Work Unit 072 Assessment of Infectious Diseases of Military Importance

Investigators.
Principal: COL Richard N. Miller, MC

Associate: LTC Ernest T. Takafuji, MC
MAJ Wayne M. Lednar, MC
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LTC Adeline Washington, ANC
Dr. Lytt I. Gardner

Objective: To assess the actual or potential impact of selected diseases of military importance. Military importance is determined by examining existing or historical morbidity and mortality data or analysis of potential threats. Studies are primarily epidemiological in nature and represent cooperative efforts with other divisions at WRAIR and USAMRDC.

Progress:

1. Disease Surveillance Associated with Jungle Training. Units continue to be monitored for infectious diseases acquired during deployments to Panama. Surveillance activities are directed at deployments with greater exposure to the elements and more intense training. During the past year, no new agents have been identified, although unexplained fevers still occur. Efforts are continuing to determine the etiologies of febrile illnesses.

2. Hookworm Infections following Deployment to Grenada. Several weeks following deployment of the 82nd Airborne Division to Panama, several soldiers with abdominal pain were noted to have significant eosinophilia on peripheral blood smear. Many of these soldiers were later confirmed to have hookworm (Necator americanus) infections based on positive stool samples. Exposure was related to the initial days of the operation when ground exposures to suburban areas near the capital city of St. George with compromised sanitation resulted in infection. Based on eosinophilia of greater than 15% and positive stools, it was estimated that over 25% of the soldiers surveyed were infected. The outbreak demonstrated the continuing threat of geohelminthic infections to U.S. military forces deployed to areas with poor sanitation.

As a result of cases of hookworm infection and strongyloidiasis resulting from jungle training and the outbreak of hookworm infection following the Grenada operation, a Soil-Transmitted Helminths Workshop sponsored by the Joint Technology Coordinating Group was held at WRAIR on 29 May 1984. The workshop was intended to address diagnostic, screening, therapeutic, and preventive issues related to geohelminthic infections. The workshop was successful in identifying shortcomings in all of these areas and the need for research.

3. Hepatitis B Infection Among U.S. Personnel in Korea. In collaboration with the Department of Virus Diseases, sera obtained from volunteers in the Gonorrhea Vaccine Study were also screened for hepatitis B surface and core antibodies. Data suggested a correlation with time assigned in Korea and acquisition of antibody. As a result of these findings, the Armed Forces Epidemiological Board recommended that U.S. forces assigned in Korea should be vaccinated with hepatitis B vaccine. Protocols are being evaluated that could assess the risk factors associated with hepatitis B transmission in Korea more clearly.

4. Penicillinase-producing and Spectinomycin-resistant Neisseria gonorrhoae infection among U.S. forces in Korea. With the adoption of the policy of treatment of uncomplicated gonococcal infections in Korea with spectinomycin, rates of gonorrhea have been carefully monitored for evidence suggesting spectinomycin resistance. Over the past 12 months, rates have remained below 25 cases/1000 men/month, similar to rates during the previous 12-month period. Overall STD rates have dropped by approximately one-third for unexplained reasons, and the incidence of syphilis has remained stable. Published reports of double resistance and recent anectodal data suggest that spectinomycin resistance may be increasing, however, with a need to double the therapeutic dose of spectinomycin in many cases. A reassessment of antibiotic sensitivity patterns of gonococcal strains is planned in the near future.

5. Korean Hemorrhagic Fever (KHF). With the continuing occurrence of cases of KHF among U.S. forces in Korea (nine cases in 1982 and three cases thus far in 1983), protocols have been developed to define the epidemiology of KHF among U.S. forces stationed in Korea. In collaboration with USAMRIID, the Division of Preventive Medicine is compiling information on the recent cases from various data sources. No preventive measures are currently being implemented in Korea.

6. Deployments to Honduras. Specific units, such as engineer units from Ft. Lewis, the Presidio of San Francisco, and Ft. Riley and Special Forces personnel have been surveyed during and following deployments to Honduras. Diarrheal disease has been identified as the most common disease problem, affecting over 50% of the soldiers surveyed. Occasional cases of malaria (P. vivax primarily) have been documented in personnel failing to take adequate prophylaxis. No cases of dengue or leishmaniasis have been identified among U.S. forces, despite high endemnicity in specific areas of deployments.

7. Rabies Prophylaxis. The death of a Peace Corps volunteer with a history of preexposure rabies prophylaxis and reports of low antibody responses among recent vaccinees has raised concern on the true efficacy of the intradermal use of current lots of human diploid cell vaccine (HDCV) for pre-exposure prophylaxis. In order to establish continued immunogenic potency of current HDCV (Merieux Institute-manufactured vaccine), twenty volunteers from the Animal Handler Advanced Individual Training Course at Forest Glen, Maryland were administered HDCV vaccine by the intramuscular route. Post vaccination serological analysis showed that all individuals achieved significant antibody titers well above what was considered protective. Geometric mean titers were not significantly different from those reported in earlier trials of HDCV given by the IM route.

A surveillance program is being conducted with 1st Special Operations Command soldiers at Fort Bragg, North Carolina, who receive pre-exposure rabies immunization with the same HDCV vaccine. Approximately one hundred soldiers will have post-vaccination antibody titers documented to monitor vaccine immunogenicity after administration of HDCV by the intradermal route.

8. Acute Respiratory Disease Surveillance. The Acute Respiratory Disease (ARD) Surveillance Program was originally established to monitor efficacy of current vaccines against various agents, including influenza and adenoviruses. The system was maintained by the Department of Virus Disease until the spring of 1984, when the Division of Preventive Medicine assumed responsibility for storage, maintenance, analysis, and reporting of the program data. Information has been collected from all Army basic training posts along with epidemiologic information. Updates of computer software and hardware are underway to permit more complete and accurate data entry, analysis, retrieval, and feedback of information to the posts.

9. Streptococcal Pharyngitis as a Cause of ARD Admissions. Increases in the ARD rates among basic trainees at Fort Leonard Wood during the months of March-April 1984 accompanied by substantial increases in the number of positive laboratory reports for Group A beta-hemolytic streptococci prompted an EPICON investigation. It was apparent that carriage rates were increased in certain training units up to 35% without apparent increases in hospital admissions for ARD. The high streptococcal isolation rates appeared to be independent of ARD admissions, supporting the hypothesis the streptococci accounted for only a small portion of ARD among recruits. A prospective study was conducted to determine the etiology of ARD admissions; results are being analyzed.

10. Meningococcal Disease Among Basic Trainees. During 1982 and 1983, fifteen cases of meningococcal disease were diagnosed among active duty soldiers at Fort Benning. All but one of these cases were in trainees. Since the fall of 1982, when the tetravalent

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meningococcal vaccine was fielded for use, there were twelve cases of meningococcal disease at Fort Benning, all from one training brigade. Seven of the nine recent cases were serogrouped and serotyped and found to be Group B/Type 2b.

An EPICON investigation was conducted at Fort Benning. The brigade from which cases were resulting was billeted in modern air-conditioned buildings, while the unaffected brigade was billeted in older wooden barracks. A prospective meningococcal carriage study was conducted in companies from both brigades, and demonstrated that rates of carriage were predicted by the number of initial carriers and the specific carriage strains. No Group B/Type 2b strains were identified and no meningococcal disease occurred during the study period.

During February 1984, four cases of meningococcal meningitis were diagnosed among trainees at Fort McClellan. Three of the four case strains were serogrouped and serotyped and found to be Group B/Type P1.16. An EPICON investigation revealed an overall meningococcal carriage prevalence in the four affected companies of 53% (447/843); the prevalence of the specific disease-causing strain was 15.3% (129/843), with carriage higher in platoons to which cases belonged. Rifampin prophylaxis was apparently effective in reducing the incidence of disease.

Because of the suggestion of the barracks environment contributing to spread of respiratory disease and meningitis, environmental studies are being developed to identify environmental factors that may be contributing to ARD transmission.

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Formal Presentations:

1. "Medical Threat to Marines Deployed to the Jungle Operations Training Course", Camp Pendleton, Panama, October 1983, CPT Patrick W. Kelley.

2. "Research Endeavors in Panama - 1983", GPM Residency Program Advisory Committee Meeting, 19 October 1983, LTC Howard Weiner.

3. "Shigella Outbreak During Bright Star 83", GPM Residency Program Advisory Committee Meeting, 19 October 1983, CPT John McNeil.

4. "Infectious Disease Threats Associated with Jungle Training". Association of Military Surgeons of the U.S., San Antonio, Texas, 1 November 1983, LTC Ernest T. Takafuji.

5. "Clinical and Epidemiologic Aspects of Meningococcal Meningitis", Grand Rounds, MEDDAC, Fort Benning, GA, 7 November 1983, MAJ Wayne Lednar.

6. "Epidemiology of Meningogoccal Meningitis in Basic Trainees", Preventive Medicine Activity, 19 November 1983, MAJ Wayne M. Lednar.

7. "Epidemiology of Meningococcal Meningitis in Basic Trainees", Preventive Medicine Activity, Fort Benning, Georgia, 19 November 1983, MAJ John Brundage.

8. "Meningococcal Disease in Basic Trainees", Preventive Medicine Activity Personnel, Fort Benning, Georgia, 20 December 1983, MAJ John Brundage.

9. "Brightstar 83", Uniformed Services University of the Health Sciences, Bethesda, MD, 27 January 1984, LTC Ernest T. Takafuji.

10. "Epidemiologic Aspects of Infectious Disease Affecting U.S. Army Deployments Abroad", Military Medicine Lecture, Uniformed Services University of the Health Sciences, Bethesda, MD MS-2, 27 January 1984, MAJ Wayne M. Lednar.

11. "Leptospirosis Update", AFEB, 3 February 1984, COL Richard N. Miller.

12. "Epidemiology of Infectious Diseases", George Washington University School of Medicine, Washington, D.C., February 7 and 9, 1984, MAJ Wayne Lednar.

13. "Diarrheal Disease During Brightstar 83", U.S. CENTCOM Surgeon's Conference, Tampa, Florida, 28 February 1984, LTC Ernest T. Takafuji.

14. "Tick-Borne Encephalitis", 10th Special Forces Detachment, Europe, 2 March 1984, CPT John McNeil.

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15. "Clinical and Epidemiologic Aspects of Food and Waterborne Outbreaks During Field Exercises", 10th Special Forces Group Medical Elements, Bad Tolz, Germany, 2 March 1984, MAJ Wayne M. Lednar.

16. "Leptospirosis", Pan American Health Association, Washington, D.C., 7 March 1984, LTC Ernest T. Takafuji.

17. "Epidemiology and Prevention of Tick-borne Encephalitis in Troops Assigned to Europe", 7th Medical Command HQ Staff Heidelburg, Germany, 8 March 1984, CPT John McNeil.

18. "Epidemiology and Prevention of Tick-borne Encephalitis in Troops Assigned to Europe", 7th Medical Command HQ Staff, Heidelburg, Germany, 8 March 1984, MAJ Wayne Lednar.

19. "EPICON", 6AFS, AHS, Fort Sam Houston, Texas, 3 April 1984, CPT Patrick W. Kelley.

20. "Military Significance of Leptospirosis", Short Course on Military Veterinary Medicine, Walter Reed Army Institute of Research, Washington, D.C., 12 April 1984, CPT Patrick W. Kelley.

21. "Preventive Medicine Concerns Related to Overseas Deployment", FORSCOM Surgeon's Conference, Atlanta, Georgia, 24 April 1984, LTC Ernest T. Takafuji.

22. "Outbreak of Hookworm Infection Following the Grenada Operation", Workshop on Soil-Transmitted Helminthics, Walter Reed Army Institute of Research, Washington, D.C., 29 May 1984, CPT Patrick W. Kelley.

23. "Military Significance of Intestinal Helminthic Infections", Workshop on Soil-Transmitted Helminthics, Walter Reed Army Institute of Research, Washington, D.C., 29 May 1984, LTC Ernest T. Takafuji.

24. "Shigellosis and Brightstar 83", Preventive Medicine Symposium, Walter Reed Army Institute of Research, Washington, D.C., 5 June 1984, LTC Ernest T. Takafuji.

25. "Eosinophilia and Intestinal Helminthic Infections", Preventive Medicine Symposium, Walter Reed Army Institute of Research, Washington, D.C., 7 June 1984, CPT Patrick W. Kelley.

26. "Doxycycline Prophylaxis Against Leptospirosis", Uniformed Services University of the Health Sciences, Bethesda, MD, 19 June 1984, LTC Ernest T. Takafuji.

27. "Epidemiology Consultant Service", AFEB, Walter Reed Army Institute of Research, Washington, D.C., 21 June 1984, LTC Ernest T. Takafuji.

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28. "Evidence for Intra-Prison Transmission of Hepatitis B", Armed Forces Epidemiology Board (AFEB), Fort Detrick, Maryland, 21 June 1984, MAJ Wayne Lednar/MAJ Robert R. Redfield.

29. "Risk of Hepatitis B Associated with Assignment to Korea Among U.S. Army Troops, Armed Forces Epidemiology Board (AFEB), Fort Detrick, Maryland, 21 June 1984, MAJ Wayne M. Lednar.

30. "Preparation of Overseas Travelers", Allergy & Immunization Clinic, Walter Reed Army Medical Center, Washington, D.C., 22 June 1984.

31. "Helminthiasis: The Current Military Problem", Tropical Medicine Course, Walter Reed Army Institute of Research, Washington, D.C., 17 July 1984, CPT Patrick W. Kelley.

32. "The Military Significance of Helminthic Infection", Uniformed Services University of the Health Sciences, MPH Seminar, 25 July 1984, CPT Patrick W. Kelley.

33. "Infectious Disease Epidemiology Laboratory", Tropical Medicine Course, Walter Reed Army Institute of Research, Washington, D.C., 13 August 1984, COL Richard N. Miller, M.D., and Lytt I. Gardner, Ph.D.

34. "EPICON", Tropical Medicine Course, Walter Reed Army Institute of Research, Washington, D.C., 15 August 1984, LTC Ernest T. Takafuji.

35. "Epidemiology Consultant Service", Tropical Medicine Course, Walter Reed Army Institute of Research, Washington, D.C., 15 August 1984, LTC Ernest T. Takafuji.

36. "Infectious Disease Epidemiology Laboratory", Tropical Medicine Course, Walter Reed Army Institute of Research, Washington, D.C., 16 August 1984, COL Richard N. Miller and Lytt I. Gardner, Ph.D.

37. "Meningococcal Disease", Tropical Medicine Course, Walter Reed Army Institute of Research, Washington, D.C., 16 August 1984, MAJ John Brundage.

38. "EPICON", 6-AF5 Course, Academy of Health Sciences, San Antonio, Texas, 13 September 1984.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OG 6760	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636

3. DATE PREV SUMMARY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
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11. TITLE (Precede with Security Classification Code)
(U) Threat Assessment of Diseases of Military Importance in the Tropics

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE 81 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
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c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORK YEARS	b. FUNDS (In thousands)
84	8.0	1,259
85	8.0	986

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME US Army Medical Component, AFRIMS
b. ADDRESS (Include zip code) Washington, D.C. 20307-5100	b. ADDRESS Bangkok, Thailand
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR SODETZ, F J
d. TELEPHONE NUMBER (Include area code) (202)-576-3551	d. TELEPHONE NUMBER (Include area code) 66-2-281-7776
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	e. NAME OF ASSOCIATE INVESTIGATOR (If available) Rosenberg,R M Hoke,C H Henchal,E A Echeverria, P D Taylor,D N Webster, H K

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Volunteers; (U) RAM I; (U) Lab animals; (U) Pig (U) Malaria; (U) Diarrhea; (U) Chancroid; (U) Vectors; (U) Dengue, (U) Japanese Encephalitis (U) Hepatitis

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The technical objective is to assess the risk of various tropical diseases to military troops and operations, and to determine the potential mortality and morbidity of military personnel undertaking operations in the tropics.

24. (U) This requires defining the ecology, epidemiology, and etiology of various tropical diseases through the development of new or improved technologies related to field studies, in vitro cultivation, microbiological assays, vector colonization, serological procedures, and other necessary approaches.

25. (U) 83 10-84 09 Surveys of epidemics to detect Non-A Non-B hepatitis are continuing. A serosurvey to determine incidence of HTLV3B (AIDS agent) has begun. Japanese encephalitis studies were done in Kampangphet, and Aranyaprathet and Pak Chong to isolate the virus and acquire strains from humans, swine and mosquitoes. Sentinal pigs were placed in the communities to determine the rate of seroconversions as well as to obtain isolates from various parts of the country. Studies of malaria susceptibility and detrimental effect of infection on colony reared members of the various sibling species were continued. Differentiation of sibling species by electrophoretic, cytogenetic, cross mating and morphological characteristics continued. A new monocolonal RIA test to differentiate the species of sporozoites in wild caught mosquitoes is being field tested, and will be used in a long term study of sporozoite to gametocyte ratio in a rural village where transmission is occurring. Area wide mosquito survey and taxanomic work continues. Studies on the biochemistry of immunosuppression in malaria continue. The long term study of dengue fever as a cause of PUO at Children's Hospital continues. Evaluation of enterotoxic E. coli strains as causes of diarrhea is continuing. For technical reports see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83 - 30 Sep 84.

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EDITION OF MAR 68 IS OBSOLETE

PROJECT NUMBER: 3M162770A870 MEDICAL DEFENSE AGAINST INFECTIOUS DISEASE

Work Unit Number 073: Threat Assessment of Diseases of Military Importance in the Tropics

Investigators: COL Frank J. Sodetz, MSC; LTC Donald S. Burke, MC; LTC George S. Ward, VC; LTC Charles H. Hoke, MC; LTC Peter E. Echeverria, MC; LTC Richard G. Andre, MSC; MAJ Michael R. Elwell, VC; MAJ Horace K. Webster, MSC; MAJ George S. Childs, MSC; MAJ David N. Taylor, MC; CPT Ronald M. Rosenberg, MSC; Katchrinnee Pavanand, M.D.; Ananda Nisalak, M.D.; Rapin Snitbhan, M.D.; Chiraphun Duangmani, M.D.; Markpol Tingpalapong, VC

The Polymicrobial Etiology of Travelers' Diarrhea

OBJECTIVE: To determine the etiology of travelers' diarrhea and the frequency of isolation of multiple pathogens.

PROGRESS: In a study of travelers' diarrhea in 35 Peace Corps volunteers in Thailand, 20 (57%) volunteers had a total of 30 episodes of diarrhea during the first 6 weeks in country. Enteric pathogens were associated with 90% of the episodes of diarrhea. One pathogen was identified in 17 (57%) episodes and 2-4 pathogens were identified in 10 episodes (33%). Fourteen asymptomatic infections were identified as well.

Enterotoxigenic *E. coli* (ETEC) were identified in 37% of these episodes and different serotypes of *Salmonella* were isolated in 34% of episodes. Infections with 9 other enteric pathogens also were identified: *C. jejuni* 17%, *Plesiomonas* 13%, *Aeromonas* 10%, *Blastocystis hominis* 7%, Norwalk virus 7%, *V. parahemolyticus* 3%, Non-01 *V. cholerae* 3%, *V. fluvialis* 3%, and rotavirus 3%. Serologic studies to detect antibodies to the LT toxin of ETEC, *C. jejuni* , Norwalk virus and rotavirus were useful adjuncts to the study of travelers' diarrhea. In this study, travelers' diarrhea was caused by a wide range of enteric pathogens of which ETEC and *Salmonella* were the most common. Infections with multiple enteric pathogens and asymptomatic infections were more common than previously recognized.

FUTURE OBJECTIVE: Study completed.

Chloroquine Prophylaxis Associated With A Poor Antibody Response to Human Diploid Cell Rabies Vaccine

OBJECTIVE: To determine if chloroquine prophylaxis interferes with antibody response to intradermal rabies vaccination.

PROGRESS: This study suggests that continuous antimalaria chemoprophylaxis with chloroquine during primary immunization appears to be associated with poor antibody response to intradermal HDCV. The antibody titers of those who took prophylaxis for less than the entire immunization period (4 weeks or less) were not significantly different from those who did not take prophylaxis. Since many PCVs who took chloroquine also took Fansidar, we are not able to separate the effect of the two drugs on this association.

FUTURE OBJECTIVE: Study completed.

Antimicrobial Susceptibility and Characterization of Outer Membrane Proteins of *Haemophilus ducreyi* Isolated in Thailand

OBJECTIVE: To determine effective treatment for chancroid based on in vitro susceptibility and to characterize strains of *H. ducreyi* by outer membrane protein profiles.

PROGRESS: Strains of *Haemophilus ducreyi* isolated in Thailand from patients with chancroid were tested by the agar dilution method against 10 antimicrobial agents and typed by outer membrane pattern (OMP) using SDS gel electrophoresis. All strains produced beta lactamase and were resistant to tetracycline, kanamycin, and sulfonamides. Most had a decreased susceptibility to trimethoprim (MIC_{50} 0.5 mcg/ml) and chloramphenicol (MIC_{50} 8 mcg/ml). Strains were sensitive to ciprofloxacin (MIC_{90} 0.001 mcg/ml), ceftriaxone (MIC_{50} 0.0015 mcg/ml), erythromycin (MIC_{50} 0.015 mcg/ml), rosoxacin (MIC_{50} 0.03 mcg/ml), and spectinomycin (MIC_{50} 8 mcg/ml). The degree of antimicrobial resistance found in Thailand is higher than that reported for *H. ducreyi* isolated in other regions.

Five different OMP patterns were found by analyzing proteins in the range of 29 to 61 kilodaltons, but 98% of the Thai strains fell into 3 patterns which did not differ greatly. OMP patterns of Thai strains were also seen in strains from other geographic areas. A new OMP type was found among 9 strains isolated in Singapore.

FUTURE OBJECTIVE: Study completed.

A Comparative Study of Enterotoxigenic Escherichia coli, Shigella, Aeromonas, and Vibrio as Etiologies of Diarrhea in Northeastern Thailand

OBJECTIVE: To determine the importance of enterotoxigenic Escherichia coli , Shigella , Aeromonas , and Vibrio as etiologies of diarrhea in rural Thailand.

PROGRESS: The incidence of enterotoxigenic Escherichia coli (ETEC), Shigella , Aeromonas , and Vibrio was determined in patients with diarrhea seen at a hospital in northeastern Thailand, and compared with the incidence of these bacteria in household contacts and their neighbors. ETEC was identified in 17 percent, Shigella in nine percent, Aeromonas in nine percent, V. parahaemolyticus in five percent, and non-01 V. cholerae in two percent of 299 patients with diarrhea. These five species of bacteria were isolated more often from patients with diarrhea than persons without diarrhea ($p<0.001$). ETEC and Shigella were found more often in household contacts and neighbors of index cases than in persons living in homes not associated with these infections (22/141 and 18/147 vs 32/1318, and 3/76 and 4/93 vs 13/1437; $p<0.001$). Livestock were present at homes of patients with ETEC diarrhea more often than at homes of patients without ETEC diarrhea (12/52 vs 66/424; $p<0.025$). Water was identified as a source of Aeromonas in homes of patients with diarrhea infected with this bacteria more often than in homes of patients without Aeromonas diarrhea (6/68 vs 49/1514; $p<0.05$). Both Aeromonas and non-01 V. cholerae can be enteric pathogens; further efforts should be made to define the enteropathogenicity of these bacteria.

FUTURE OBJECTIVE: Study completed.

Non-01 Vibrio cholerae in Thailand: Homology with Cloned Cholera Toxin Genes

OBJECTIVE: To determine the prevalent of genes coding for cholera toxin in non-01 V. cholerae in Thailand.

PROGRESS: Two hundred and eighty-one non-01 Vibrio cholerae isolated in Thailand were examined for homology with genes coding for cholera toxin (CT). Five isolates from environmental sources were homologous with the CT gene probe and produced both the A and B subunits of CT.

FUTURE OBJECTIVE: Study completed.

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Prevalence of Heat-stable II Enterotoxigenic *Escherichia coli* in Pigs, Water, and People at Farms in Thailand as Determined by DNA Hybridization

OBJECTIVE: To determine the prevalence of heat-stable II enterotoxigenic *Escherichia coli* on pig farms in Thailand.

PROGRESS: The DNA hybridization assay employing a 460 base pair fragment of DNA encoding for ST-II enterotoxin was used to determine the prevalence of heat-stable II enterotoxigenic *Escherichia coli* (ETEC) in pigs, people, and water at 57 farms in Sri Racha, Thailand. ST-II ETEC was found in 62 (3%) of 2110 suckling, 181 (32%) of 560 weaned, and 4 (1%) of 457 adult pigs examined. Twenty one percent of 62 suckling pigs with ST-II ETEC infections, but none of 185 infected older pigs had diarrhea. ST-II ETEC was found more frequently in suckling pigs with diarrhea than without diarrhea (13/146 vs 49/1946 $p < 0.001$). ST-II ETEC was detected in water collected from three of 57 clay jars containing water used to bath at three pig farms, in one jar used to bath immediately after working in the barn, and from one farmer who did not have a recent history of diarrhea. Evidence of this organism was not found in 245 other individuals living on the pig farms, or in 220 inhabitants and 114 water specimens collected at tapioca farms nearby. In Thailand ST-II ETEC was found in suckling pigs with diarrhea, but was infrequently found in man.

FUTURE OBJECTIVE: Study completed.

Identification by DNA Hybridization of Enterotoxigenic *Escherichia coli* in a Longitudinal Study of Villages in Thailand

OBJECTIVE: To determine the incidence of enterotoxigenic *Escherchia coli* in villages in Thailand over one year employing DNA hybridization techniques.

PROGRESS: Radiolabeled genes for enterotoxins were used to study the epidemiology of enterotoxigenic *Escherichia coli* (ETEC) infections in Thai villages. When *E. coli* isolated from 674 specimens were fixed on nitrocellulose paper and examined for hybridization with *E. coli* enterotoxin gene probes in Bangkok this technique had a sensitivity of 94 percent (31/33) and a specificity

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clinical diagnosis of chancroid. For men with H. ducreyi culture-positive ulcers, cure rates were 100% in 25 men treated with ceftriaxone, 87% in 23 men given TMS for 7 days and 55% in 31 men given TMS in a single dose. For men with H. ducreyi culture-negative ulcers cure rates were 100% in 29 men treated with ceftriaxone, 66% in 32 men given TMS for 7 days and 63% in 24 men given TMS in a single dose.

H. ducreyi was isolated on follow-up examination in 17 (46%) of 37 men who were treatment failures and none of 127 men who were cured. H. ducreyi was reisolated in 4 (29%) of 14 men who failed on TMS for 7 days and 13 (57%) of 23 men who failed on TMS in a single dose. The mean inhibitory concentration of ceftriaxone for 50% ($MIC_{50\%}$) of 94 H. ducreyi strains was 0.004 mcg/ml. The $MIC_{50\%}$ for trimethoprim was 16 mcg/ml before treatment and greater than 32 mcg/ml in 18 strains isolated after treatment was begun. All H. ducreyi strains were resistant to sulfamethoxazole. Ceftriaxone in a single dose of 250 mg is effective; but TMS, even when given as a standard 7 day regimen, is not effective treatment for chancroid in Thailand.

FUTURE OBJECTIVE: Study completed.

Serosurvey and Virus Isolation From Rodents to Determine the Hantaan Virus Presence in Thailand

PROBLEM: Recent studies have found Hantaan virus to be the causative agent of Korean hemorrhagic fever (KHF), a disease syndrome of potential military significance in Korea and Manchuria and of potential significance in the USSR, the Balkans, parts of Western Europe and Scandinavia.[1] Evidence has recently been obtained in Seoul, Korea that urban Rattus are chronically infected with Hataan virus.[1] Cases of KHF in man have now been linked to infected wild rats in urban Seoul and Osaka, Japan. In addition, antibodies to Hantaan virus have been found in Rattus captured near the docks in Japan, Korea and United States.[3] Chronic infection of rats and international shipping provide a chain which may have disseminated this virus worldwide. Therefore the potential for this agent to cause human disease may be far greater and more widespread than is presently appreciated.

OBJECTIVES:

1. To identify areas in Thailand where rodents have antibody for Hantaan virus.

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of 100 percent (641/641) compared to testing E. coli for enterotoxin production in the Y-1 adrenal and suckling mouse assays. However, when the same specimens were fixed directly on nitrocellulose paper at a field laboratory and transported to the reference laboratory for assay with the gene probes 27 specimens which contained ETEC did not hybridize with the E. coli gene probes.

ETEC that hybridized with the LT, ST-H, and ST-P probes was identified in ten percent (17/177) of villagers with diarrhea, seven percent (8/108) of contacts of individuals with ETEC diarrhea, and three percent (32/1199) of persons not associated with ETEC diarrhea. ETEC that hybridized with the ST-II probe was not a cause of diarrhea. Alternative methods of retaining DNA on filters under field conditions are needed before this technique can be used to examine specimens directly with enterotoxin gene probes.

FUTURE OBJECTIVE: Study completed.

Colonization Factors Associated with Enterotoxigenic Escherichia coli in Thailand

OBJECTIVE: To determine the colonization factors associated with enterotoxigenic Escherichia coli in Thailand.

PROGRESS: Eighty-six percent (72/84) of LTST, none of 141 LT, and 24 percent (27/111) ST ETEC isolated in Thailand aggregated in less than one molar $(NH_4)_2SO_4$, hemagglutinated human group A and bovine erythrocytes in one percent d-mannose, and possessed either CFA I or CFA II. No other colonization factors were identified by these two methods.

FUTURE PROGRESS: Study completed.

Comparative Study of Ceftriaxone and Trimethoprim-Sulfamethoxazole for the Treatment of Chancroid in Thailand

OBJECTIVE: To determine the optimal treatment of chancroid in Thailand.

PROGRESS: Single dose ceftriaxone, 250 mg intramuscularly, was compared to trimethoprim-sulfamethoxazole (TMS), 160/800 mg by mouth twice daily for 7 days, or TMS, 640/3200 mg by mouth in 1 dose for the treatment of chancroid in men in Thailand. Haemophilus ducreyi was isolated from 79 (48%) of 164 men with a

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2. To test human sera from areas with rodent infection to determine if there is serological evidence of human infection.

3. To isolate Hantaan virus from tissues of rodents in endemic areas.

PROGRESS: In 1983 we reported the first serological evidence of Hantaan or Hantaan related virus in Thailand. A high percentage of antibody titers to Hantaan(24%) was found in bandicoots as well as in people (33%) living in the vicinity of the trapped rodents at a village near Kanchanaburi. Personnel from the department of Virology returned to the village at a later date. Sera were obtained from new and those persons previously tested. No antibody was detected in serum of new personnel, however a decrease in antibody level was noted in those that had been previously positive. This could indicate the infection had been recent.

All results are not complete but to date virus isolates have been made from 3 rats and 2 bandicoots. Serological characterization of these isolates is in progress. Titers from the additional 94 animals bled have not been determined. The department of virology completed a retrospective and prospective study in the Kanchanaburi provincial hospital. It was determined that all hemorrhagic fever cases seen at Kanchanaburi were due to dengue.

FUTURE OBJECTIVES:

1. Finish testing sera and tissues on hand and reconfirm results.
2. Look for evidence in nephrology patients that Hantaan virus infection might have caused renal failure.

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2. Ho Wang Lee, Luck J Baek and Karl M. Johnson: Isolation of Hantaan virus, the Etiologic Agent of Korean Hemorrhagic Fever, from Wild Urban Rats. In press.

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Letter to the editor. NEJM 307:624.

Incidence of Leptospirosis in Eastern Thailand

PROBLEM: Leptospirosis is a common zoonotic disease found throughout the world. The clinical features in man range from an influenza-like illness to a more severe disease form manifested by continued fever with meningitic symptoms and signs. In some cases infection can lead to renal and hepatic failure, jaundice, and even death. Leptospirosis is frequently found in the tropical areas of the world and recent attention has focused on several outbreaks in soldiers training in jungle areas. Symptomatic treatment and antibiotic therapy are used in the acute illness. However, once symptoms are evident the beneficial effect of antibiotics is questionable. The relatively long recovery period, even with treatment, suggest that prevention is the practical approach in solving the problem of leptospirosis. It is difficult to prevent direct contact with leptospira contaminated water in a tropical environment, especially during military maneuvers. Immunization against specific serovars of leptospira can be protective. However the serovars as identified by new methods need to be isolated and characterized if vacine potential is to be considered.

OBJECTIVES:

1. Determine incidence of leptospirosis in Thai military patients on duty near Kampuchea which have fever of unidentified origin.

2. Determine Leptospirosis incidence of suspect patients in the Prachinburi Provincial Hospital.

PROGRESS: Blood cultures to detect active leptospiremia were taken on FUOs during screening of Thai military personnel for malaria. Of 118 cultures there was only 1 positive. This serovar was identified as icterohaemorrhagiae and pyrogenes by the microagglutination test(MAT). Since MAT was positive on both serovars the culture was sent to CDC for specific identification. Blood cultures as well as acute and convalescent serum specimens were taken from suspect leptospirosis patients at the Prachinburi hospital. Five of 37 blood cultures were positive. The serovars isolated were bataviae(4) and tabaquite(1).

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FUTURE OBJECTIVES:

1. Continue screening military FUOs but obtain a representative sampling of serum to determine if presence of antibody contributes to the relatively low incidence.

2. Continue culturing serology of patients at the Prachinburi hospital plus determine possible sources or reservoirs in one high incidence area identified in FY 84.

3. Determine if the ELISA test developed at WRAIR is effective for rapid diagnosis of suspect patients at Prachinburi hospital.

Leptospirosis Incidence in Bangkok Animal Reservoirs

PROBLEM: Dogs, cattle and rodents are susceptable to leptospirosis. Leptospirosis can result in severe illness or chronic renal disease in animals and they may be a source for human infection when organisms are shed into the environment. Although Bangkok is an urban environment there are many dairy cattle, for local milk consumption, as well as some beef cattle maintained within the city. Dogs and rodents are commonly encountered on the streets and sois of Bangkok. During the rainy season, the chance of human and animal contact with leptospira contaminated flood waters is increased. In a previous study of military war dogs in Northeast Thailand during the rainy season, only 2 of 214 tested dogs had antibody to leptospira. Within Bangkok, cases of human leptospirosis are common in the flood season, and almost all are the result of the bataviae serovar infection.

If canines are an important source of the zoonotic leptospira infection in Bangkok, appropriate vaccination could prevent this disease in dogs and reduce shedding of the organisms into the environment. However, due to lack of cross immunity between serovars, the specific serovar or serovars that produce canine infection must be determined. This is particularly true if bataviae is the infecting serovar, as it has little antigenic relationship to other leptospira serovars.

The incidence and serovar types present in Bangkok animals which can serve as a reservoir for human infection is unknown. In a small preliminary survey we found that bataviae is the most common serovar infection of rodents in Bangkok. Determination of the serovars present in Bangkok can be accomplished by culturing blood and urine from cattle, rodents and dogs. This epidemiological data will be used for determining what serovars exist and providing valuable information for possible control measures.

OBJECTIVES:

1. To determine by serology and/or isolation the serovars of Leptospira in Bangkok cattle, dogs and rodents.

2. To compare the incidence of canine leptospirosis infection iin Bangkok before and after the flood period.

PROGRESS: Data is complete from the post-flooding canine survey. Two hundred sixty two canine sera were tested by microagglutination test(MAT). Fifteen serovars were detected, the 3 most common were *bataviae* , *robensoni* and *louisiana* respectively. Antibody presence increased with age. Young dogs had 6%; juveniles 23%, and in adult dogs, positive leptospira titers were present in 54%. Two dogs had active Leptospirosis, (#1497) was infected with *bataviae* and the other (#1513) was infected with more than one serovar.

From 12 Jun to 14 Aug 84, 204 blood and 150 urine specimens were collected from dairy cattle in Bangkok. Serum specimens were tested by MAT for the presence of antibody. Twelve serovars were detected. The 3 most prevalent were *bataviae* , *tabaquite* and *hepdomadis.* Fifty four of 204 cattle sera were positive by MAT. Nineteen of these contained more than one serovar. There was an increasing incidence with age. Four, 9 and 41% positive sera were detected in calves, heifers, and cows, respectively. Leptospira antibody was detected in 7 of 8 amphors in Bangkok. Bataviae was the most widely distributed serovar, being present in all 7 of the positive amphors. Cultures from 150 urine specimens are being processed but results are not yet available.

A preliminary rodent survey has been completed, however numbers and sites need to be expanded.

FUTURE OBJECTIVES:

1. Trap and process 200 rodent specimens.

2. Analyze data after present samples have been processed and 200 more rodents have been added to the study.

Leptospirosis in the Non-Human Primate Model

PROBLEM: Leptospirosis is a common zoonotic disease found throughout the world. The clinical features in man range from an influenza-like illness to a more severe disease form manifested by continued fever with meningitic symptoms and signs. In some cases

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infection can lead to renal and hepatic failure, jaundice, and even death. Leptospirosis is frequently found in the tropical areas of the world and recent attention has focused on several outbreaks in soldiers training in jungle areas. Symptomatic treatment and antibiotic therapy are used in the acute illness. However, once symptoms are evident the beneficial effect of antibiotics is questionable. The relatively long recovery period, even with treatment, suggest that prevention is the practical approach in solving the problem of leptospirosis. It is difficult to prevent direct contact with leptospira contaminated water in a tropical environment, especially during military maneuvers. Immunization against specific serovars of leptospira can protect animals but immunization of man is not practical unless the serovar endemic to the area is identified or a vaccine with broad antiserovar activity is developed. Last year we reported that the experimental infection of monkeys with a local human isolate of the bataviae serovar produced a bacteremia of one to six days, infection of the CSF, and a bacteruria for up to four weeks. An antibody response was detected by microagglutination by one week and peak titers were reached by 3-4 weeks.

OBJECTIVE:

1. To characterize clinical leptospirosis in the non-human primate model.

2. To determine the efficacy of antibiotic treatment as a prophylaxis for the acute infection.

3. To determine if an ELISA method for detecting leptospira antibody or antigenemia is a useful means for obtaining rapid early diagnosis of leptospirosis.

PROGRESS: During FY 84 it was determined that Doxycycline prophylaxis was effective in the hamster and rhesus models of leptospirosis. A short in vitro study to determine if resistance might develop to doxycycline was completed. No evidence of resistance development was observed.

FUTURE OBJECTIVES:

1. Further develop and refine the ELISA to detect low antibody titers to leptospira.

2. Test sera from monkeys immunized with different serovar antigens by the ELISA method and find an antigen or combination of

antigens that will detect serovar infections that are present in Thailand.

Japanese Encephalitis Virus Seroconversion of Pigs in Northeast Thailand

PROBLEM: Pigs are thought to be the amplifying host for JEV. Studies on pig seroconversion rates in Chiangmai, Japan, and Sarawak have shown high rates of seroconversion that are not constant throughout the year. Chiangmai is an area with a high human attack rate. A study at CQ farm near Korat will allow calculation of seroconversion rate in a province with an intermediate attack rate. This information will help in determining the importance of swine in JE transmission and possible differences in various locations in Thailand. Infection in pigs are a means for monitering the JE season. Increased seroconversion in pigs is seen when JE cases occur in man. However, in Southern Thailand, there are many pigs with JE antibody and no reported cases of JE in man. Multiple factors may account for this including climate and vector differences and differences in strains of virus. Attempts will be made to isolate virus from these pigs so it can be compared with recent isolates from an area where clinical JE infection in man is common.

OBJECTIVE: To determine the monthly JEV seroconversion of pigs in Northeast Thailand.

PROGRESS: The testing of pigs for HAI antibody to JEV began on 15 June 1983 and was completed 15 June 1984. One hundred thirteen 3 month old pigs were bled. Twenty five had positive HAI titers and were not studied further. Seroconversion occurred throughout the year at a low level (8-30%). From the results available, 54 pigs have seroconvereted. The average monthly seroconversion had been 35% (range 8-69%). An effort to isolate JE virus from JE HAI neg sera which were collected one month before seroconversion failed. The JE pig IgM antibody capture ELISA (MAC) testing of convalescent sera alone detected 61% (17/28) of JE infection as defined by HAI seroconversion, although there was only moderate probability of a positive MAC in the convalescent specimen. In the pig the JE viremia lasts only 72 hours. A MAC positive test is a marker for infection within preceding two weeks (Burke, unpublished data, note CQ farm pig serology, 1984). Fifty four suspicious sera have been cultured but no virus replication was detected in any culture. This was probably due to improper serum storage since CQ had no ultralow temperature capacity.

FUTURE OBJECTIVE: Analyze data and complete report.

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Biochemistry of the Malaria Parasite

PROBLEM: Resistance to all clinically proven antimalarial drugs has been documented for *P. falciparum.* This includes the new drug, meflqouine, a quinolinemethanol. In addition, there is now evidence for possible cross-resistance to the aminoalcohol compounds, halofantrine and enpiroline, which are presently undergoing clinical trials. Therefore, the prospect of new antimalarial drugs for the prophylaxis and treatment of multi-drug resistant *P. falciparum* malaria is dampended by the likelyhood of parasite resistance even before the drugs are introduced for large scale usage. This situation may be explained in part by the structural similarity of the new drugs to quinine, an unfortunate consequence of empirical drug development. Thus there is an urgent need to discover and include novel chemical structures in the design of new antimalarial compounds. While some relief may come from natural products (e.g. the herbal remedy, quinghaosu) major relief will only come through rational drug development. Rational approaches, however, are seriously hampered by a lack of knowledge concerning the malaria parasites basic biochemistry.

PROGRESS: We have sought to systematically study the intermediary metabolism of the major human malaria pathogen, *P. falciparum,* and two experimental monkey malarias, *P. knowlesi* and *P. cynomolgi.* Emphasis has been given to purine, pyrimidine and folate metabolicm in an attempt to identify parasite unique metabolic targets.

Human malaria parasite adenosine deaminase. Human malaria infected erythrocytes show a dramatic increase in adensoine deaminase activity *in vitro.* Using continuous culture techniques, adenosine deaminase-deficient human erythrocytes were infected *in vitro* with the major human pathogen *Plasmodium falciparum.* Adensoine deaminase activity was undetectable in the uninfected host red cells, but increased by 2-fold over normal levels in these cells with an 8% parasitemia. The enzyme in these cells appeared unique in that its activity was markedly elevated over that of other parasite purine enzymes, was not cross-reactive with antibody against human erythrocyte adensoine deaminase, and though inhibited competitively by deoxycoformycin was relatively insensitive to erythro-9-(2-hydroxy-3-nonyl) adenine. The use of adensoine deaminase-deficient erythrocytes for the *in vitro* cultivation of *Plasmodium* provides a unique system for the study of parasite enzyme and allows further insight into the purine metabolism of the intraerythrocytic malaria parasite.

Guanosine-5′-triphosphate cyclohydrolase in Plasmodium: Evidence for De Novo synthesis of folate cofactors. GTP cyclohydrolase (E.C. 3.5.4.16), the first enzyme in the pteridine pathway leading to the *de novo* formation of folic acid, has been identified and isolated from the human malaria parasite, *P. falciparum.* The enzyme was purified 200-fold by high performance size-exclusion chromatography (HPSEC) using a TSKG-3000 SW protein column. The molecular weight was estimated at 300,000. Optimal enzyme activity was observed at pH 8.0 and at 42°C. Products of the enzyme reaction were identified as the carbon-8 of GTP and D-erythrodihydroneopterin triphosphate. The Km for GTP was 57 uM. ATP was a competitive inhibitor (Ki of 600 uM) of the enzyme. Activity of the enzyme was Mg^{2+}-independent, whereas Mn^{2+}, Cu^{2+} and Hg^{2+} (5 mM) were inhibitory. GTP cyclohydrolase activity was also identified in a murine malaria parasite, *P. berghei,* and a simian parasite, *P. knowlesi.* Activity of the enzyme in *P. knowlesi,* an intrinsically synchronous quotidian parasite, was found to be dependent on the stage of parasite development.

Purine strategy of the intraerythrocytic (IE) malaria parasite. Through the use of labelled precursors and specific enzyme inhibitors we have developed a perspective on the intermediary metabolism of the IE malaria parasite regarding purines both *in vitro* and *in vivo.* Basically, the IE parasite acquires purines by salvage of purine bases from its environment and not by *de novo* synthesis. *In vitro,* the parasite utilize hypoxanthine for synthesis of purine nucleotides (ATP and GTP) and nucleic acids (DNA and RNA). Conversion of hypoxanthine to ATP occurs via a parasite unique pathway involving the intermediate adenylosuccinate and two enzymes, adenylosuccinate synthetase and adenylosuccinate lyase. *In vitro* the situation appears different for the IE parasite. The parasite has a very active adenosine deaminase (ADA) which acts on adenosine to produce inosine which in turn is acted on by purine nucleoside phosphorylase to produce hypoxanthine. Inhibition of ADA *in vivo* with the inhibitor deoxycoformycin (DCF) knocks out synthesis of purine nucleotides and greatly reduces the parasitemia in *P. knowlesi* infected rhesus monkeys. However, DCF at the doses used *in vivo* does not eliminate infection. The IE parasites survive because of plasma hypoxanthine which is directly salvaged. Nonetheless, *in vivo* the parasite appears to acquire hypoxanthine primarily by catabolism of adenosine. The importance of these observations is that the design of specific purine inhibitors cannot be focused on a single salvage enzyme. It appears thus that effective inhibition of purine metaboism in the IE malaria parasite will require a combination of specific inhibitory agents.

Immune Cell Changes in Malaria Infection

PROBLEM: The rational development of a malaria vaccine requires an understanding of the mechanisms involved in protective immune responses to malaria. Basically host immunity to natural malaria is poorly developed. Studies in our laboratory have been directed at understanding the fate of peripheral blood mononuclear cell (MNC) populations in acute malaria infection. In particular, we have sought to examine the underlying cellular biochemistry associated with the functional disturbances in the immune system during acute infection - especially, immunosuppression.

PROGRESS:

Changes in Mononuclear Cells from Thai Gem Miners Working Along the Thai-Kampuchean Border.

The Thai gem miners represent a unique study population in that they are adults from non-malarious areas experiencing repeated falciparum malaria infection as a result of their occupation. Peripheral blood mononuclear cells (MNC) obtained from individuals with acute falciparum malaria showed changes in cell number and distribution, responsiveness to mitogens and metabolism of adenosine 3′, 5′-monophosphate (cAMP). Patients were adult males with a history of 2-4 recent episodes of malaria. Controls were adult males with no history of malaria. MNC were isolated by density-gradient centrifugation, cell subsets identified by monoclonal antibodies and response to mitogens measured by $[^3H]$ thymidine incorporation. cAMP was measured by radioimmunoassay. Compared to healthy controls, malaria patients were lymphopenic with decreased proportion and absolute number of T lymphocytes (OKT 3+), T helper (OKT 4+) and T suppressor (OKT 8+) cells. Natural killer cells (Leu 7+) were decreased in absolute number whereas the proportion of HLA-DR+ cells was increased. Malaria MNC showed a depressed proliferative response to phytohemagglutinin, concanavalin A and pokeweed mitogen. Endogenous cAMP levels were decreased in malaria MNC. When control MNC in buffer (37°C) were exposed to adenosine (20 uM) a spike in cellular cAMP production occurred within 10-15 minutes. The adenosine mediated cAMP response was greatly suppressed in malaria MNC. These observations suggest a biochemical defect in the second messenger role of cAMP in host MNC which may underlie changes in immune function associated with malaria infection.

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Studies with P. cynomolgi and P. knowlesi Infected Rhesus monkeys

Given our observations on humans with acute falciparum malaria we sought to verify the MNC cAMP defect in an animal malaria model. In both P. cynomolgi and P. knowlesi infections rhesus MNC were observed to have a defect in cAMP metabolism during the acute phase of infection. This MNC defect, however, was reversed during convalescence (i.e., once the parasites were eliminated from the peripheral blood by chloroquine treatment). The defect was characterized by decreased intracellular cAMP levels in MNC and by decreased responsiveness to adenosine stimulation of cAMP production. The biochemical changes correlated functionally with a reduction in the proliferative response of rhesus MNC to concanavalin A.

At the present time we are working to understand the biochemical basis for the MNC cAMP defect in acute malaria infection. It is known that adenosine receptors are associated with the regulatory subunit of membrane adenylate cyclase. Therefore, adenosine plays an important-though poorly understood-role in cAMP metabolism. We have observed a decrease in plasma adenosine levels in both human and monkey acute malaria infection. The alteration in host adenosine appears to be associated with an exceptionally active parasite enzyme, adensoine deaminase. We are currently studying detailed aspects of host MNC cAMP and purine metabolism both in freshly isolated MNC and by co-culture of host MNC with parasitized erythrocytes. In addition, we are working to identify the cAMP defect in specific T lymphocyte sub-sets.

RECOMMENDATIONS: These are the first studies to identify a biochemical defect in immune cells in malaria infection. The presence of an alteration in cyclic AMP metabolism provides a valuable insight into how functional impairment of immune effector cells may arise in acute malaria. Perhaps more importantly identification of a biochemical defect suggests the prospect of pharmacological intervention to restore or prevent changes in cellular immune function that may interfere with the host response to a malaria vaccine. This work should be continued.

Mosquito Survey and Taxonomic Studies

PROBLEM: To elucidate the mosquito fauna of Thailand and Southeast Asia. Primary emphasis is put on the determination of diagnostic characters that identify that belong to species complexes.

PROGRESS: During the past year, morphological studies were continued on the sibling species of the *balabacensis* and the *maculatus* complexes. These studies are being done in collaboration with the WRAIR Biosystematics Unit at the Smithsonian Institution. Manuscripts are in preparation to describe new species in the *balabacensis* complex, with the members currently being refered to as *Anopheles dirus* A, B, C, or D. Discriminating morphological characters have been found that will differentiate some of the new species in the *maculatus* complex. A key has been prepared and is included in a manuscript still under preparation (1). Work on the *Aedes (Finlaya)* manuscript continues with over 22 plates having been completed. Progeny rearings of vector species from many locations in Thailand and several locations in West Malaysia have been completed and correlated with genetic identifications.

FUTURE OBJECTIVES: Taxonomic studies on members of the *balabacensis* and *maculatus* complexes will continue. Morphological characters to discriminate field-collected *An. dirus* A, B, C, and D will be tested with populations in Thailand and Malaysia. The key to separate the *maculatus* complex also will be tested in the field with large population samples. The final ten plates for the *Aedes (Finlaya)* manuscript will be prepared.

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Mosquito Genetics

OBJECTIVE: Define the specific taxa in the *Anopheles balabacensis* and *An. maculatus* species complexes using cytogenetic and electrophoretic techniques.

BACKGROUND: Past work done by us (eg. WRAIR Ann. Rpt., 1982) has shown that both *An. balabacensis* and *An. maculatus* , once thought to be single species, are in fact complexes of sibling species. In general, the adult stages are morphologically identical. Because both complexes contain the most important malaria vectors in SE Asia a thorough study of specific characteristics as determined by cytogenetic, enzymologic, and biological factors is warranted. The ultimate goal is to be able to identify all the members of each complex, map their ranges, and determine their vectorial capacities.

PROGRESS: Of particular importance was the finding of further evidence, from cross breeding experiments, that dirus C and dirus D are true species. In a comparative study the polytene chromosomes of F-1 larval salivary glands from dirus D exhibited striking and unique fixed inversions in the X chromosome and in the banding sequences at the tips of arms 2L and 2R (Baimai et al., in prep.). Forced crosses of An. dirus A and dirus C, which are closely related and sympatric, produced fertile hybrids of both sexes in one direction but sterile F-1 males in the opposite direction. Crosses of dirus B with A, C, and D produced either no hybrid F-1 generation or one with completely sterile males. An. dirus D showed similar genetic incompatibility with A, B, and C and with balabacensis s.s. from Sabah as well. And in cytogenetic studies, dirus A, which shows little chromosomal inversion polymorphism, was found to exhibit marked variation in constitutive heterochromitin around the sex chromosome centromeres (Baimai et al. 1984). Three types of X and 2 types of Y have been found so far in wild population samples. Analyses of metaphase chromosomes of An. dirus A, B, and C, and An. takasagoensis using the Hoechst 33258 fluorescent staining technique have revealed remarkable differences in fluorescent banding patterns of the sex chromosomes, particulary the Y chromosomes of these taxa. These differences are mainly in the amount and distribution of heterochromatin.

Concerning An. maculatus, the most important finding was proof that maculatus G is a fourth species of the complex. It has two unique inversions fixed on the Xa arrangement and a further two on the stephensi arrangement of arm 2. All four maculatus species have been found feeding on water buffalo at Pak Chong, about 220 Km NE of Bangkok. Adult characteristics that allow the visual differentiation of adult females with 90% certainty were found and a key produced, which is now being field tested.

FUTURE OBJECTIVES: Large samples from natural populations of both species groups from different regions will be differentiated using as criteria the cytogenetic and morphological characteristics so far found. Once the range of each species is determined work on specific biological differences can begin. This work will also serve to support the development and testing of DNA probes, explained elsewhere (ILIR, 3M161101A 91C DA 303116).

Highly-efficient, Dry Season Malaria Transmission in Southeastern Thailand

OBJECTIVE: To correlate, for the first time, species ratios of Plasmodial sporozoites in mosquitoes with that of gametocytes in humans in an area of natural transmission.

BACKGROUND: Although it is relatively easy to identify mosquitoes infective for malaria, there is no way to visually distinguish one species of human *Plasmodium* from another or, often, from non-human species. Therefore it has been implicitly assumed in mathematical models of malaria transmission that the ratio of *Plasmodium* species being introduced into a community by mosquito bite is the same as the ratio in gametocytes in that same population. There is reason to suspect this presumption is wrong: many *Anopheles* mosquito species differ in their susceptibility to different species of malaria. With the possible advent of malaria vaccine the accuracy of transmission models becomes increasingly important. In order to correlate gametocyte ratios with sporozoite species identified immunologically we found it necessary to establish a longitudinal study.

PROGRESS: The first year of a two year longitudinal study has been completed. Blood was examined monthly from everyone in a small stabile, prosperous farming community in Southeastern Thailand. Also monthly, seven all-night, man-baited collections of *Anopheles* mosquitoes were done by 2 men at each of 2 sites in the village. All cases of malaria uncovered by examining 100 fields of a thick film were treated (quinine-tetracycline for *P. falciparum*).

The study population (for each of whom at least 8 monthly peripheral blood films were examined) was 180, 49% of whom were aged under 20 yr. During 12 months 67% had at least one episode of *P. vivax* and 96%, one of *P. falciparum*; 98% had either one or the other. The ratio of *P. falciparum* to *P. vivax* was approximately 3 to 1. Gametocytes occurred in 13% of *P. vivax* and 7% of *P. falciparum* cases. Cases were usually symptomless: only 7.5% of the population complained of fever during the 3 weeks preceding blood collection and, of these, only 45.3% had demonstrable parasitemia. No clustering of *P. falciparum* cases was seen, possibly because this disease is so prevalent, but there was a significant clustering of *P. vivax* cases in two small groups of neighboring houses that may be due to the resting habits of the vector or possibly multiple feeds by a disturbed, infective vector.

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The only *Anopheles* species found infective was *An. dirus.* A total of 866 were caught biting man during 12 months, of which 19 or 2.2% were infective. Theoretically, each person in the village received 23 infective bites a year or about one every 16 days. In fact, transmission was markedly seasonal. Populations were high with parity rates low during the late rainy season (May-Oct) and low with high parity during the dry season. In October, 350 *An. dirus* were caught but parity was 45% and none were infective. In February only 35 were caught in 28 man-nights but 74% were parous and 6, or a phenomenal 17%, were infective. IFA identification of sporozoites using antisera raised in rabbits to local sporozoites found 20% of infective mosquitoes carried *P. vivax.* Statistical correlation with gametocyte ratios is not yet complete.

These preliminary results suggest that previously holoendemic, asymptomatic malaria foci are more common in Thailand than previously suspected and probably play an important role in the country's malaria epidemiology.

FUTURE OBJECTIVES: The second year of the study is underway. It is identical to the first year except that *P. falciparum* is treated with Mefloquine-Fansidar and that certain physiological parameters (eg. spleen rates, leucocyte counts, hematocrits) will be included. At the end of year II we will be able to evaluate the efficacy of the new drug regimen on an entrenched focus.

Detrimental Effects of Plasmodial Infections on the Survival Rate ofAnopheles dirus

PROBLEM: The objectives of this study are as follows: a. to determine if the longevity of mosquitoes infected with *Plasmodium* is different to a significant degree from that of uninfected mosquitoes; b. to determine if the longevity among mosquitoes with heavy or light infection rates is significantly different; and c. to determine if the longevity of mosquitoes infected with different species of *Plasmodium* is different significantly among groups.

PROGRESS: An investigation of the effects of human malaria parasites on the longevity of *Anopheles dirus* continued this year. Over one hundred and fifty lots of mosquitoes have been fed on *Plasmodium vivax* patients. One hundred and ten lots of mosquitoes were allowed to feed on *P. falciparum* patients. About 40% of the feeds were positive. Control lots of mosquitoes were fed on uninfected volunteers on the day of the patient feed. Survival of control mosquitoes and lightly infected mosquitoes was excellent, with some mosquitoes living more than seventy days. Heavily infected mosquitoes usually died within one month. A manuscript on

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the correlation of survival rates of *An. dirus* with different infection densites of *P. cynomolgi* is in press.

FUTURE OBJECTIVES: Data from these experiments will be entered into the computer for statistical analysis. Feeds will continue to be made during the next year to increase the number of infected comparisons.

Identification of Field-collected Sporozoites

PROBLEM: Four different tests are available to potentially identify *Plasmodium* sporozoites in mosquito salivary glands: a. circum-sporozoite precipitin test; b. immunofluorescent antibody test; c. radioimmuno assay test; and d. enzyme-linked immunosorbent assay test. The objectives of this study are to provide *Plasmodium falciparum* and *P. vivax* sporozoites to WRAIR for the development and improvement of these tests, to evaluate these four tests in the laboratory with mosquitoes infected with Thai strains of *Plasmodium*, and to adapt the tests for use in the field to identify natural infections in vector anophelines.

PROGRESS: Sporozoites were routinely harvested from salivary glands dissected from *Anopheles dirus* infected with *P. falciparum* or *P. vivax* 14d after being fed on human gametocyte carriers at our Kanchanaburi field station (see "Detrimental effects of *P. falciparum* on *An. dirus* survival", WRAIR Ann. Rpt. 1983-84). For some tests the rate of infectivity was determined by dissection of a sample of 20 and the rest of the mosquitoes killed by freezing, air dried, and stored at room temperature. A preliminary field test of an ELISA for *P. falciparum* developed from Brazilian sporozoites was done at our Chantaburi field site in November 1983 and indicated that the test was strain specific. Therefore, BALB/C mice were inoculated with live sporozoites of each species (initial inoculum of 10^6/mouse; 4 boosters of 10^5 each) and sent to Dept. of Entomology, WRAIR where fusions and separations of monoclonals were done. Initial testing of specificity was also done at WRAIR using sporozoites on slides and dried infective mosquitoes supplied by us. Consequently, ELISA's for both *P. falciparum* and *P. vivax* have been developed that are species but not strain sensitive (see publication: Burkot et al. 1984).

FUTURE OBJECTIVES: Continued evaluation of the ELISA's against Thai strains is needed, both from our western Thailand field station and from other regions. We have obtained human-use permission to collect sporozoites from eastern Thailand and intend eventually to field test the ELISA on wild caught mosquitoes (see "Highly efficient, dry season malaria transmission in Southeastern Thailand", this report).

Ectoparasite and Rickettsia tsutsugamushi Studies in Thailand

PROBLEM: The objectives are to establish and describe ectoparasites that are potential vectors of human pathogens in Thailand; and to determine the distribution of natural populations of larval mites infected with *Rickettsia tsutsugamushi* in Thailand.

PROGRESS: A checklist of the ticks occuring in Thailand has been revised and published (1). The Genus *Miyatrombicula* was redefined and a new species was described (2). Illustrations of new species of the Genus *Leptotrombidium* have been prepared and a manuscript is in preparation. Collections of potentially infected chiggers were conducted at Khao Yai, Sakarat, and Pak Thong Chi. The chiggers were sent to USAMRU in Malaysia for attempted colonization and determination of infectivity status.

FUTURE OBJECTIVES: Because several new species of *Leptotrombidium* have been discovered and were found to carry *R. tsutsugamushi,* a collaborative study with the USAMRU Lab in Kuala Lumpur is being planned. This study would determine the role these new species play in the transmission of scrub typhus. Chromosome studies of *L. deliensis* also are proposed to determine if this species occurs in Thailand or if it is a sibling species and possibly not important in disease transmission.

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Dengue Viruses from Dengue Haemorrhagic Fever Cases by Mosquito Inoculation and Using Three Mosquito Cell Lines

PROBLEM: Dengue Haemorrhagic Fever (DHF) is one of the principal causes of child morbidity and mortality in S.E. Asia principally in Thailand, Vietnam, China, Burma and Indonesia (Halstead, 1984). Despite the high prevalence of the disease the isolation of dengue viruses from these patients has always been difficult and time-consuming. Over the last decade improvements in isolation success have been mainly as a result of the use of intrathoracic inoculation of mosquitoes. Rosen and Gubler (1974) concentrated attention on the inoculation technique when they described the isolation of dengue viruses inoculated into *Aedes albopictus* mosquitoes. Later, they substituted the much larger mosquito *Toxorhynchites amboenensis* because of its safety and better survival (Quoted in Tesh, 1979). *Toxorhynchites splendens* mosquitoes have also been used (Watts *et al*, 1982) and the technique has been developed to intra-cerebral inoculation of these mosquitoes (Thet Win, 1981) and to inoculation of larvae (Pang *et al*, 1983) resulting in more rapid reading of results. In all cases viral antigen was detected after inoculation by immunofluorescence.

In contrast, it is surprising that although several dengue virus-sensitive mosquito cell lines are available, very little material has been published on dengue virus isolation in mosquito cell lines, particularly from DHF patients (for a bibliography see Kuno and Flores, 1982). Laboratories without entomological facilities may prefer the flexibility that a tissue culture system offers.

OBJECTIVE: To compare the isolation of dengue viruses from DHF patients using the three most widely used mosquito cell lines namely the C6/36 clone (Igarashi, 1978) of Singh's *Aedes albopictus* cell line, the *Aedes pseudoscutellaris* (LSTM-AP-61) cell line established by Varma *et al* (1974) and the *Toxorhynchites amboenensis* (TRA-284-SF) cell line adapted to serum-free medium (Kuno, 1982).

PROGRESS: The results of dengue virus isolation using a mosquito inoculation technique on specimens obtained from Dengue Haemorrhagic Fever patients at the Children's Hospital, Bangkok, for 1982 and part of 1983 are described. 77 selected plasma samples, which had previously yielded 39 dengue virus isolates, were inoculated onto three virus-sensitive mosquito cell lines and the isolation success compared. Isolation was markedly dependent on the haemagglutination inhibition (HAI) antibody titre of the plasma. Estimates of the isolation success rate if cell cultures had replaced the mosquito inoculation technique suggest that in samples with HAI titres $\leq$ 320 the *Toxorhynchites amboenensis* (TRA-284-SF) cell line would have an isolation success of 52% compared to 43% on *Aedes pseudoscutellaris* (LSTM-AP-61) cells, 21% on *Aedes albopictus* (C6/36) cells compared to the observed 61% by mosquito inoculation.

RECOMMENDATIONS:

1. The TRA-284-SF cell had the highest overall isolation rate but was also the most difficult of the three lines to maintain. This cell line obviously warrants much more extensive evaluation.

2. The LSTM-AP-61 cell line had quite a high isolation rate and was easy to handle with a moderate growth rate and very good attachment to both glass and plastic surfaces. Under field isolation conditions the LSTM-AP-61 cells have a proven record of being robust cells able to be transported long distances unattended, inoculated under difficult field conditions and requiring the minimum of attention over long incubation periods. On balance, it would seem to be the system of choice for field-orientated isolation work.

3. The C6/36 cells had a surprisingly low isolation rate which could not be explained on the basis of an unhealthy stock of cells as parallel field isolations of Japanese encephalitis virus using these cells has yielded a large number of strains. The C6/36 cells grow very quickly and are very easy to handle but they tend to exhaust the tissue culture medium rapidly making extended incubations difficult. The C6/36's over-riding attribute is that, being a cloned cell line, results should be consistent in widely dispersed laboratories. This makes this line ideally suited for laboratory based studies on the detailed biological and biochemical events in arbovirus replication *in vitro* .

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Dengue hemorrhagic fever (DHF) at Bangkok Children's Hospital, 1962-1984

PROBLEM: Persons throughout the tropics are at risk of dengue infection. A particularly severe form of dengue, hemorrhagic fever, occurs primarily in southeast Asia. Persons having multiple infections are felt to be at highest risk, since almost all patients have serological responses suggestive of prior infection. Knowledge of the risk of sequential infections has led directly to the decision to develop tetravalent dengue vaccines, rather that monovalent vaccines, which might leave immunized persons at risk of

DHF. In development of vaccines for dengue, it is vital to maintain surveillance of etiology. Both Thailand and the U.S. Army are developing such vaccines. This study provides vital data for both efforts.

OBJECTIVE: To determine the incidence of dengue hemorrhagic fever (DHF) and the serotype of dengue virus causing DHF in patients at Bangkok Children's Hospital (BCH).

PROGRESS: During the 22 years of this study, a total of 4474 patients have been diagnosed as having DHF (Table 1). About 20% of these have yielded a dengue virus isolate. 62% of all isolates were D2, 18% D1, 14% D3 and 6% D4. The highest mean number of cases occurring in August (40 cases average). Because of several quiet years since 1980, a marked increase in activity of DHF was predicted in last year's report. In 1984, the number of cases occurring thus far has been second only to the number in 1980. As of October 1, 1984, cases continue. Dengue 2 is once again the most frequent isolate, however, dengue 3 has appeared in a substantial fraction of the cases. Following the occurrance of a large epidemic of chikungunya in Indonesia, chikungunya has reappeared in Bangkok after several years of absence.

Dengue isolates from DHF patients, 1962-October, 1983

YEAR	TOTAL CASES	DEN ISOL	D1	D2	D3	D4	% D1	% D2	% D3	% D4	MAJOR TYPE
1962	148	50	17	23	9	1	34	46	18	2	D2/1/3
1963	156	35	8	10	17	0	23	29	49	0	D3/2/1
1964	333	105	29	53	20	3	28	50	19	3	D2/1/3
1965	88	12	0	8	3	1	0	67	25	8	D2/3
1966	55	10	7	1	0	2	70	10	0	20	D 1
1973	135	22	5	13	4	0	23	59	18	0	D2/1/3
1974	151	21	8	7	6	0	38	33	29	0	D1/2/3
1975	399	14	1	8	5	0	7	57	36	0	D2/3
1976	176	9	0	6	1	2	0	67	11	22	D2/4
1977	495	66	0	37	10	19	0	56	15	29	D2/4
1978	185	33	0	28	1	4	0	85	3	12	D2
1979	301	61	2	58	0	1	3	95	0	2	D2
1980	788	240	50	174	14	2	21	73	6	1	D2/1
1981	196	36	11	21	3	1	31	58	8	3	D2/1
1982	206	25	4	17	1	3	16	68	4	12	D2/1
1983	400	53	3	25	15	10	6	50	30	20	D2/3
1984	414*	28	0	17	7	4	0	61	25	14	D2/3
TOTAL	4478	820	145	506	116	53	18%	62%	14%	6%	D2

* Specimens processed through October

RECOMMENDATION: This study is recognized as the longest continuous DHF surveillance activity. The data from it is invaluable in guiding the Army's efforts to develop a dengue vaccine. Moreover, it has become the centerpiece of a collaborative study of DHF between the military laboratories in the region. The study should be continued, with modifications appropriate for the expected increase in DHF activity.

Dengue fever at Bangkok Children's Hospital, 1984

PROBLEM: Dengue was the most common cause of undifferentiated fever in U.S. Troops in Vietnam[1]. Dengue fever continues to occur in epidemics ever summer throughout Thailand. To maintain surveillance of the magnitude and etiology of dengue fever, a longitudinal surveillance system was established several years ago in which selected children presenting with undifferentiated fevers at the Bangkok Children's Hospital are evaluated for the presence of dengue.

OBJECTIVE: To determine the relative importance of dengue virus as the etiology of undifferentiated fevers in children in Bangkok.

PROGRESS: The outpatient clinic of the Children's Hospital, Bangkok is visited each weekday. Blood specimens and clinical information are collected from a sample of children with fevers. From 10 to 14 days later, follow-up specimens are collected. Acute specimens are cultured for dengue virus. HAI serology for dengue is done on each serum.

This study has been in progress since 1979, excluding 1982 (Table 1). during that time, a total of 679 cases were evaluated. 29% of the fully evaluated cases had evidence of flavivirus as the etiology of fever.

Table 1. Dengue Isolations and Serology from Cases of Undifferentiated Fever at Bangkok Children's Hospital, 1979-1983.

YEAR	CASES EVALUATED	SEROTYPE OF ISOLATES 1	2	3	4	TOTAL ISOL	SEROCONVERSIONS PRIMARY	SECONDARY	NOT FLAVI
1979	166	1	8			9	1	10	155
1980	263	38	25	4	1	68	42	52	169
1981	35	4	4				5	4	26
1982	Not done								
1983	126	1	1	3	6	11	9	32	81*
1984	87	NOT COMPLETE					9	32	40**
Total:	679	44	34	7	7	92	67	129	471

* Chikungunya seroconversion, 4 cases ** Chik seroconversion 6 cases

Overall, dengue 1 has been the predominant isolate, in contrast to the predominance of dengue 2 in DHF patients. Most of the isolates occurred in 1980, a year in which a record number of DHF cases and isolates occurred. In that year, 36% of children cultured yielded a dengue isolate, attesting to the fact that much of the increased load of febrile children was due to the dengue epidemic.

CONCLUSION:

1. 25% of undifferentiated fevers in children are attributable to flavivirus infections, presumably dengue.

2. Dengue 1 and 2 are the most frequent isolates, accounting for 94% of isolates.

3. In contrast, dengue 2 alone, rather than dengue 1 is a relatively more common isolate from DHF cases seen at the same hospital.

RECOMMENDATION: This study represents a long term surveillance of dengue isolates in patients with a clinical syndroime likely to be seen in US troops affected by epidemic dengue. Moreover it allows longitudinal determination of serotypes of dengue virus causing dengue fever. Comaprison of these data with those from the DHF study will allow determination of the relative importance of dengue serotypes in the two syndromes. The longitudinal study should be continued and expanded to include more epidemiological evaluation.

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Development of Human Monoclonal Antibodies to Dengue Virus

PROBLEM: In a previous series of experiments we showed that the "Traffic" in the peripheral blood of specific dengue antibody producing cells increases markedly during acute dengue virus infections in humans. [1] Specific IgM production is readily detectable in in vitro cultures of Ficoll-Hypaque gradient purified peripheral blood mononuclear leukocytes (PBML) obtained during the first 3 days of hospitalization, while specific IgG production in in vitro PBML cultures is detectable in cells obtained during the first 7 to 10 days of hospitalization and is of much greater magnitude. We reasoned that specific dengue hybridomas could be prepared by fusion of continuous lymphoblastoid cells with PBML cells obtained from dengue patients early in their hospital course.

OBJECTIVE: To produce monoclonal human dengue enhancing antibodies in order to understand the mechanisms of antibody dependent immune enhancement of dengue virus growth in macrophages and other cells with FC receptors.

PROGRESS: Human PBML abtained during the early "B cell burst" were fused with a hypoxanthine phosphoribosyl transferase (HPRT)-deficient human lymphoblastoid cell line (UC729-6)[2]. After several preliminary unsuccessful attempts, a fusion protocol was developed that produced a viable fusion product. Seven logs of PBML were fused with an equal number of UC 729-6 cells. in 40% PEG 8000 for 15 minutes at 26 degrees c. After fusion, 100,000 cells were plated in each of 96 well microtiter plate without feeder layers. The post-fusion media was HAT containing RPMI 1640 supplemented with 20% fetal calf serum. Clonal out growth usually occurs between three to four weeks. The clones are tested for immunoglobulin production in supernatant fluid by a simple sandwich ELISA. The producing clones were expanded and subcloned by limiting dilutions. It was found that the stability of the sub-clones, especially IgG producers was poor. In general, they have continued to secrete immunoglobulin over a two to three month period. Some of them were difficult to subclone by limiting dilutions.

A total of 26 fusions have been performed. 53 stable fusion products have been obtained, of which 46 secreted IgG and 7 secreted IgM. None reacted directly with dengue viruses. The specificity of these clones remains to be determined.

RECOMMENDATION: The stable clones have been tested for binding to dengue virus surface glycoprotein (V3)[3]. None were reactive. However, V3 represents only 17% of all the dengue genome coding capacity. The cell supernatants should be tested for their capability to bind to other viral specific proteins.

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Determinants of the Outcome of Japanese Encephalitis

PROBLEM: Japanese encephalitis is the most common cause of arboviral encephalitis in the world today (1). Although much is known about the complex zoonotic life-cycles of this viruse, relatively little is known about the pathogenesis of infections in the human host. Only one of every 300 persons infected with Japanese encephalitis virus (JEV) develops clinical encephalitis, and among these 20 to 40% die (2,3). In northern Thailand, epidemics of Japanese encephalitis recur yearly between June and August with an average of 1500 to 2000 hospital admissions and 200 to 500 deaths (4). Young males appear to be at the greatest risk of severe disease (2). However, the virologic and immunologic bases of increased risk remain undefined. Recent development of a mosquito cell line sensitive for isolation of JEV (5), along with the development of immunoassays for JEV-specific IgM and IgG responses in serum and CSF (6-8) now makes possible a direct analysis of virus-antibody interactions in human flavivirus encephalitis.

OBJECTIVE: To identify those risk factors present at the time of hospital admission which were associated with a fatal outcome in patients with acute Japanese encephalitis.

PROGRESS: Forty-nine consecutive patients with laboratory-confirmed acute Japanese encephalitis were studied to identify risk factors present at hospital admission which were associated with a fatal outcome. Sixteen patients (33%) died. The following constellation of findings correlated with a fatal outcome: (1) infectious virus in cerebrospinal fluid (CSF), (2) low levels of Japanese encephalitis virus-specific IgG and IgM in both CSF and serum and, (3) a severely depressed sensorium. Age, sex, days ill before admission, distance from home to the hospital, past medical history, CSF protein content, and CSF leukocyte count were not significant risk factors.

These results suggest that a vigorous immunoglobulin response, both systemically and locally within the central nervous system, is an important factor favoring survival in acute Japanese encephalitis.

RECOMMENDATIONS: Japanese encephalitis represents a serious threat to non-immune indigenous populations, travellers and military forces operating in Asia. This laboratory is the leading laboratory in the world in the study of the pathogenesis and diagnosis of Japanese encephalitis. Evaluation of optimum methods of treatment and prevention of this disease should be pursued as high priorities to the unprecedented opportunities presented to advance our knowledge in these areas.

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Virus Isolations from Mosquitoes Collected During a Japanese Encephalitis Virus Epidemic in Northern Thailand

PROBLEM: Epidemics of human encephalitis caused by the mosquito-borne flavivirus Japanese encephalitis (JE) virus occur annually in the north of Thailand with some of the highest attack rates in the world (Hoke, unpublished data). Previous epidemiological studies were carried out in the Chiang Mai valley in the late 1960's [1-6] and in 1982 [7-10]. We report here the results of virus isolation from mosquito pools, as part of a combined hospital and field study with the aim of obtaining JE virus isolates from human encephalitis patients, porcine hosts, and mosquitoes during the 1982 epidemic period.

OBJECTIVE: To compare virus isolation using the C6/36 *Aedes albopictus* cloned mosquito cell line [11] with detection of viral antigen in mosquito pools by an antigen capture enzyme immunoassay (EIA) method and to compare rapid typing of virus isolates by indirect immunofluorescence (IFA) and EIA assay using specific monoclonal antibodies with a standard plaque neutralisation method.

PROGRESS: From 16 June to 15 August 1982, CDC light traps baited with and without carbon dioxide were used to collect mosquitoes in the province of Kamphaengphet, N Thailand. A total of 353042 mosquitoes comprising 59 species were sorted and 345173 mosquitoes were pooled for virus isolation. Virus strains were isolated from 63 pools using C6/36 *Aedes albopictus* mosquito cell cultures. These comprised 35 Japanese encephalitis (JE) virus strains, 18 Tembusu (TEM) virus strains and 3 untyped flaviviruses, 3 isolates were identified as strains of Getah (GET) virus, being the first report of the isolation of this alphavirus from Thailand, and 4 isolates remained unidentified. Most of the virus isolates were obtained from *Culex tritaeniorhynchus* mosquitoes in carbon dioxide baited light traps. JE virus was isolated only over a ten day period and the last isolate was obtained 1 week before the peak of admission of human encephalitis cases at Kamphaengphet provincial hospital. An antigen capture enzyme immunoassay (EIA) test successfully identified about 50% of the JE virus positive pools, but the method saved considerable processing time. Rapid screening of isolates grown on LSTM-AP-61 *Aedes pseudoscutellaris* mosquito cells by indirect immunofluorescence using flavivirus group-specific and JE-specific monoclonal antibodies showed a high degree of correlation with plaque reduction neutralisation tests.

RECOMMENDATION: The development of immunoassay methods for screening insect pools for viruses represents an important advance in our ability to perform field studies of the ecology of arbovirus diseases. Studies to improve the methods reported here should be pursued and applied to determining methods to reduce transmission of mosquito borne disease.

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Improved Surveillance ofJapanese Encephalitis by Detection of Virus Specific Igm in Desiccated Blood Specimens

PROBLEM: Effective programs for disease surveillance and control require the availability of accurate yet simple and inexpensive laboratory diagnostic methods. Japanese encephalitis is a disease problem of epidemic proportion in Thailand (1). In 1974, the Ministry of Health of the Royal Thai Government instituted a new nation-wide surveillance system for this disease. In this system, filter paper strips are saturated with patient blood, allowed to dry, and afixed to a standardized report form which is mailed to the Virus Research Institute of the Ministry of Public Health in Bangkok. The blood is eluted from the filter paper strips, and the eluates are examined for a rise in titer between the acute and convalescent samples by hemagglutination inhibition (HAI) assay. In a trial using specimens obtained from dengue-infected children, HAI titers obtained using serum or filter paper eluates were shown to be essentially identical(2).

Although this system has worked well for the surveillance of JE in Thailand, one problem has been that a four fold or greater rise in titer has been detected in less than 50% of the reported clinical cases of encephalitis (3). In those cases failing to show a four fold rise in HAI titer, the etiology of the encephalitis has remained undetermined. Two main possibilities exist: either (1) the

HAI test as performed on the filter paper specimens is relatively insensitive and most of the cases are in fact due to JE, or (2) there are other important unrecognized etiologies of acute encephalitis in Thailand.

Recently we devised simple solid phase immunoassays for the independent measurement of JEV specific IgM or IgG in patient blood or cerebrospinal fluid (4-6).

OBJECTIVE: To compare the results of testing filter paper eluates by antibody capture ELISA with results obtained by the more conventional HAI method.

PROGRESS: An IgM antibody capture type enzyme linked immunoassay (MAC ELISA) was compared to the hemagglutination inhibition method (HAI) for establishing a laboratory diagnosis of acute Japanese encephalitis virus (JEV) infection using specimens of dried blood eluted from filter paper strips. Paired samples from 243 encephalitis patients were tested.which had been obtained by mail through a national surveillance program in Thailand. During the peak of the 1983 encephalitis epidemic, 72% of cases were diagnosed as JE by MAC ELISA, compared to only 38% by HAI. During non-epidemic periods, the proportions diagnosed as JE by MAC ELISA or HAI were 26% and 33%, respectively. Detection of IgM anti-JE by the antibody capture immunoassay is superior to the HAI method for establishing a diagnosis of acute JE using dried blood specimens.

RECOMMENDATIONS: The results of this study have led us to conclude that the MAC ELISA approach probably has superior specificity and sensitivity when compared to the HAI test for screening of filter paper eluates to establish a diagnosis of acute JE. The MAC ELISA also compares favorably to the HAI with respect to reproducibility, cost, and ease of performance. Therefore, the technology reported here should be transferred to laboratories charged with performing surveillance of JE in Thailand.

In addition, similar tests should be developed for hepatitis A and B to facilitate surveillance of those diseases.

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Japanese Encephalitis Immunoglobulin M Antibodies in Pig Sera

PROBLEM: Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus which causes epidemics of human encephalitis throughout Asia (1,2); in Thailand swine are the major intermediate amplifying host (3). Although infected adult swine typically exhibit no signs of illness, JEV has been documented to cause abortions and fetal wastage when pregnant sows are infected (4). Currently available methods for diagnosing acute JEV infection in swine are not ideal; the most practical approach requires the demonstration of a rising antibody titer in paired sera obtained several days apart. An improved method for the serodiagnosis of JEV in swine would be useful (1) to monitor transmission rates in the major amplifying host so as to better define the risk of human disease and (2) to assess the economic impact of JEV on swine reproduction. We previously developed an immunoasssay for JEV IgM antibodies in human sera and cerebrospinal fluid and found that an early and rapid diagnosis could be made by detection of virus-specific IgM (5-7). The recent preparation of mouse monoclonal antibodies to porcine mu chain (8) now makes possible a similar approach to the rapid diagnosis of JEV in swine.

OBJECTIVE: To develop a solid phase immunoassay for the detection of swine IgM antibodies to JEV.

PROGRESS: An antibody capture enzyme-linked immunoassay was developed for detection of porcine IgM antibodies to Japanese encephalitis virus (JEV). IgM antibodies in sera were captured onto the solid phase of microtiter plates previously sensitized with mouse monoclonal antibodies to porcine mu heavy chain. JEV antigen binding to the lawn of IgM was quantitated by subsequent binding of peroxidase labelled human hyperimmune anti-JEV IgG which in the final step catalyzed a substrate color change. In sucrose density gradient fractionated sera from recently infected pigs, the peak of JEV IgM activity detected by immunoassay corresponded to the peak of 18S, 2-mercaptothanol sensitive hemagglutination inhibiting (HAI) antibody activity. Within 2-3 days, of viremia, JEV-infected sentinel pigs developed high levels of JEV IgM which then waned within 2 weeks. Among specimens collected from 99 random swine at abatoirs in Thailand during a period of low JEV transmission, none of 25 JEV HAI negative sera had JEV IgM activity, seven of 74 JEV HAI positive sera did have JEV IgM activity, and the remaining sixty-seven sera had readily detectable JE HAI antibodies but lacked JEV IgM. The JEV IgM antibody capture immunoassay is a useful test for rapidly diagnosing active or recent (within two weeks) JEV infections in swine.

RECOMMENDATIONS: The objective of this study has been achieved. The rapid diagnostic test developed herein should be applied for field surveillance of Japanese encephalitis.

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Intense Transmission of Japanese Encephalitis Virus to Pigs in a Region Free of Epidemic Encephalitis

PROBLEM: Japanese encephalitis virus (JEV), an arthropod-borne flavivirus transmitted by rice-field breeding mosquitoes of the genus Culex, is a cause of major epidemics of encephalitis throughout most of Asia from Japan to India (1). However, the geographic distribution of JEV is not limited to the epidemic region; JEV has also been isolated from mosquitoes in Malaysia, Indonesia, and the Philippines (2-3), all countries where epidemic Japanese encephalitis has not been reported (5). In Thailand, encephalitis epidemics are confined to the northern region of the country (6),where a dramatic increase of hospital admissions for acute encephalitis occurs annually during the months of June, July, and August. No such increase is observed in the south. There are no obvious reasons for this difference.

Pigs are thought to be the main amplifying host of JEV in Thailand. Most adult swine raised in northern Thailand have serum antibodies to JEV, and JEV-seronegative sentinel pigs set out during the epidemic season rapidly seroconvert. However, no published information is available on JEV transmission to pigs in the "encephalitis-silent" southern region.

OBJECTIVE: To determine whether JEV transmission to pigs occurs in southern Thailand.

PROGRESS: Epidemic Japanese encephalitis recurs annually in the northern provinces of Thailand, but human encephalitis is rare in the southern provinces. We investigated transmission of Japanese encephalitis virus (JEV) to pigs in southern Thailand. Blood specimens from 100 young pigs at abattoirs in three southern provinces were tested for JEV hemagglutination inhibiting (HAI) antibodies. Seventy-four percent were positive. Ten seronegative sentinel pigs were placed at 5 locations in the southern province of Choomporn. Seven of the 10 pigs developed JEV HAI and JEV IgM ELISA antibodies within 2 weeks of placement. JEV was isolated from all 7 seroconverting sentinel pigs from blood specimens collected 3 to 11 days after placement. Fifteen light-trap mosquito collections at the 5 locations all included known JEV vectors, some in large numbers. We conclude that there is intense transmission of JEV to pigs in southern Thailand despite the rare occurrence of human encephalitis in the same region.

RECOMENDATIONS:

1. Compare the Choomporn JEV isolates with strains collected in northern Thailand.

2. Perform field studies of the immune status of the Choomporn human population to JEV and dengue.

3. Of potentially great importance is the possibility that isolates from a region free of human disease may represent naturally attenuated variants which could serve as a live virus vaccine for man or pigs. Attenuation of these viruses should be pursued.

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Kinetics of Japanese Encephalitis Virus Immunoglobulin m and g Antibodies in Human Serum and Cerebrospinal Fluid

The Kinetics of JE Virus Immunoglobulins M and G
in Serum and CSF

PROBLEM: Japanese encephalitis virus is a mosquito-borne flavivirus which causes major epidemics of acute encephalitis in humans throughout Asia; thousands of cases are hospitalized annually (1). We recently developed an "antibody capture" type solid phase immunoassay for detection of Japanese encephalitis virus antibodies in patient sera and CSF (4,5), and in a preliminary trial found that the assay could establish an early and rapid etiologic diagnosis. It is important to further refine our knowledge of the kinetics of antibody production in order to learn when the JE MAC ELISA should optimally be done. Furthermore, measurements of perturbations of the antibody response would be valuable in evaluating therapeutic modalities which might become available.

OBJECTIVE: The objective of this investigation is to answer the following questions about the kinetics of JEV antibodies in serum and CSF: (1) How soon after onset of illness are antibodies detectable in CSF? (2) How long do detectable JEV IgM and JEV IgG activities persist in serum and CSF? (3) Are JEV antibodies detectable in the CSF of persons experiencing asymptomatic JEV infections?

PROGRESS: A prospective study of Japanese encephalitis virus (JEV) antibodies in serum and cerebrospinal fluid (CSF) was conducted during an encephalitis epidemic in northern Thailand. Antibodies were measured by hemagglutination inhibition (HAI) and by "antibody capture" solid phase enzyme linked immunoassays for JEV specific IgM or IgG (JEV MAC ELISA or JEV GAC ELISA). Thirty-two patients who met criteria for a clinical diagnosis of acute viral encephalitis had serum and CSF obtained within 12 hours of hospital admission and again 7, 30, and 180 days later. The proportions of HAI confirmed cases with detectable CSF JEV IgM antibodies on days 1, 7, 30, and 180 were 68, 100, 96, and 72% respectively. For CSF JEV IgG antibodies the proportions were 47, 89, 100, and 100% respectively. Specific anti-JEV activity of both IgM and IgG antibodies in CSF was almost always greater than in simultaneously obtained serum, as indicated by a two-fold or greater intensity of the final color reaction in the AC ELISA. Twenty-five CSF samples were obtained from control patients with other diseases with possible nervous system involvement (but none with a clinical diagnosis of viral encephalitis); none had detectable JEV IgM activity. 48% of these 25 control CSF specimens did have detectable CSF JEV IgG activity, but in no case was the JEV specific activity in CSF greater than that in simultaneously obtained serum. CSF and serum samples from five asymptomatic JEV infected siblings of encephalitis cases were tested by JEV MAC ELISA; all had high levels of JEV IgM antibodies in serum but none had detectable JEV IgM in CSF.

RECOMMENDATIONS: The increasing sophistication of this laboratory in dissecting the fine points of the immune response to JE should be capatilized on to evaluate various modes of therapy likely to be of benefit.

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Isolation of Japanese Encephalitis Virus Strains from Clinical Specimens Using a Continuous Mosquito Cell Line

PROBLEM: In Northern Thailand there is a pattern of recurrent annual encephalitis epidemics with large numbers of cases attributed to the mosquito-borne flavivirus Japanese encephalitis (JEV).[1,2] Since 1982, our field studies have examined mosquito vectors, transmission risk factors and clinical features of human infection in Kamphaengphet Province, which has one of the highest encephalitis incidence rates in N.Thailand. However, systematic evaluation of methods to optimize virus isolation methods are required. This investigation is designed to study these methods.

OBJECTIVE: To carry out a field trial of JEV isolation from clinical specimens using the *Aedes pseudoscutellaris* mosquito cell line, which is sensitive to JEV. (3,4)

PROGRESS: During the 1983 Japanese encephalitis virus epidemic in the province of Kamphaengphet, North Thailand, 16 fatalities occurred amongst 49 confirmed cases at the provincial hospital. Inoculation of fresh cerebrospinal fluid onto the *Aedes pseudoscutellaris* (LSTM-AP-61) mosquito cell line, with detection of viral growth by immunofluorescence using monoclonal antibodies, yielded an unexpectedly high virus isolation rate (5/15). Numerous virus isolates were also obtained from different areas of the brains of all seven autopsied patients, whereas virus isolation on samples obtained using a needle biopsy were successful only once in four attempts. We conclude that this mosquito cell system proved useful for virus isolation under field conditions, and that immunofluorescence was a more reliable screening technique than observation for cytopathology.

RECOMMENDATION: The methods defined by this paper allow increasing rates of virus isolation from patients with Japanese encephalitis. These methods should be capitalized on to evaluate the virological response to various therapeutic interventions, particularly dexamethasone, which is widely used but may have an effect on viral replication, and interferon which may have a positive effect by suppressing viral replication. The effects of these two drugs on the presenced of cultivable virus should be evaluated.

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Japanese Encephalitis: Immunocytochemical Studies of Viral Antigen and Inflammatory Cells in Seven Fatal Cases

PROBLEM: Japanese encephalitis (JE) virus is the commonest cause of arthropod-borne human encephalitis worldwide. The recent development of the JE MAC ELISA in this labloratory has allowed the early identification of JEVE victims early in the course of their illness. Virus isolation has been facilitated by mosquito cell lines that can be used under field conditions. No satisfactory treatment has been devised.

The histopathology of JE was carefully described in the older Japanese literature (reviewed by Miyake and Shiraki). Pathological studies in American journals appeared following outbreaks on Guam and Okinawa and among soldiers in Korea. Since those studies were reported, however, more sophisticated techniques to detect the localization of viral antigen and the presence of T cells of various classes, B cells and macrophages have been developed. These techniques can be used to develop a composite picture of the response to infection of the brain by JEV.

OBJECTIVE: To determine correlations between the pathology, distribution of virus in brain tissue, presence of viral antigen in neurons, and cellular and humoral response to fatal JEV infection.

PROGRESS: Immunocytochemical studies were performed on the brains of 7 children who died during the 1983 epidemic of JEVE in Kampangphet province, Thailand. The children died at various stages of illness. The results of study of their brains allowed formulation of the following composite pathological picture:

Normal brain shows no viral antigen in neurons and no mononuclear cells outside of vessels. Within vessels cells stain with B and T cell markers can be found, and monocytes (macrophage precursers) show esterase activity. In early cases (3 days) viral antigen is not prominent. Sensitized T cells (presumably helper /inducer T cells) cross vascular endothelium, release lymphokines and attract monocytes which differenciated to macrophages. At 4 and 5 days many neurons are laden with viral antigen. Perivascular cuffs contain T and B cells and macrophages. Macrophages and some T cells move into the parenchyma. At 6 and 8 days all neurons with antigen are surrounded by inflammatory cells and neurophagia is evident. Proportions of B and T cells and macrophages remain similar in perivascular cuffs but large numbers of macrophages and some T cells are found in many areas of grey matter. At 9 days neurons with viral antigen are rare. Only ghost-like remnants and fragments of stained dendrites are found. Nodules of macrophages contain granles of antigen. B cells remain localized to perivascular cuffs.

Thus, the infection is a straight forward invasion of neurons followed rapidly by the response of the host. Unfortunately, widespread destruction of neurons may have already occurred by the time the immune response is mounted, resulting in the inexorable downhill course seen in many patients. The extensive necrosis of neurons explains why neurological deficits may be permanent and of a variety of manifestations.

RECOMMENDATIONS: This study suggests that therapeutic interventions to inhibit viral replication early in the course of hospitalization may have a profound effect on the ultimate outcome of infection. One such agent is interferon, a non-specific inhibitor of viral replication which has in vitro activity against JEV. Clinical studies of interferon in JE are urgently needed.

Localization of Japanese Encephalitis Viral Antigen in Artificially Infected CULEX tritaeniorhynchus Mosquitoes by Avidin-Biotin Immunoperoxidase Staining

PROBLEM: Studies on the tropisms of arboviruses in mosquitoes have previously involved artificial infection of vectors followed by dissection and individual titration of organs or by cryostat sectioning and localising viral antigen using fluorescent antibody techniques. Dissection and titration is a tedious procedure of limited sensitivity and fluorescent antibody prepartions have only a short life span. Developments in immunocytochemical techniques for the detection of individual human immune cells suggested that this technique might be applicable to virus-vector studies.

OBJECTIVE: Apply immunocytochemical techniques to determine the anatomic and physiologic effects of viral infection of vector mosquitoes.

PROGRESS: Culex tritaeniorhynchus mosquitoes were collected engorging on buffalo at Bang Pa-In north of Bangkok and were allowed to lay eggs. F1 mosquitoes 3-5 after emergence were inoculated intrathoracically with approximately 1pfu (on LLC-MK2 cells) of Japanese encephalitis virus and were then held at 26 or 32 C and 85% relative humidity. At varying times after infection groups of 10 mosquitoes were frozen at -70 C for subsequent assay. In each group 5 mosquitoes were titrated individually for virus content on LLC-MK2 cells and the remaining 5 were fixed for two hours in ethanol, processed overnight in a Tissue-Tek embedding machine, and then embedded in paraffin. Standard 4-5 micron sections were cut and stained immediately by an avidin-biotin immunoperoxidase staining technique using a high titred mouse hyperimmune serum as the initial antigen labelling system. Although very sensitive some endogenous peroxidase activity was detected in early experiments which required careful adherence to the staining schedule to minimise such effects. Sensitivity aside, the principal advantage over other methods was that preparations were permanent and required only the use of a light microscope for repeated and extended observation.

Overall, a marked difference was noted in the rate of replication of JE virus at the 2 temperatures. At 26 degrees C peak titres approaching 6 logs of pfu per mosquito were not achieved until 10-14 days after infection whereas at 32 degreesC peak titres were reached as early as 4 days after infection. Although whole body titration detected virus in mosquitoes on day 1 after infection staining was not consistently positive until day 2. From day three onwards marked involvement of the cells of the compound eye were noted with subsequent massive involvement of the nervous system. At 32 degrees C salivary glands were positive as early as 4 days after infection whereas at 26 degrees C the glands were not clearly positive before 17 days after infection. Other organ involvement included the pericardial cells of the heart and occasionally areas of the oviduct, but did not apparently include the ovarian follicles.

RECOMMENDATION: These results of these studies are highly relevant epidemiologically and this staining technique is a powerful new tool which now needs be applied to other virus-vector combinations.

Field Trial of a Japanese Encephalitis Diagnostic Kit

PROBLEM: Japanese encephalitis virus (JEV) is a major cause of epidemic and sporadic cases of encephalitis throughout much of Asia (1). Most patients are admitted to provincial hospitals lacking sophisticated laboratory facilities, and are managed without benefit of a definite etiologic diagnosis. National epidemiologic surveillance systems do serologically document acute JEV infections in a small proportion of cases in some countries (2), but always long after the acute illness.

Patients with acute Japanese encephalitis produce virus-specific antibodies within their cerebrospinal fluid (CSF) (6) early during the clinical course of encephalitis (3); one diagnostic strategy is to detect intrathecal virus-specific IgM. Although the total concentration of IgM in CSF is low, the specific anti-JEV activity of IgM in CSF is high compared to that of serum; CSF specimens from patients with Japanese encephalits typically produce strong positive reactions when tested by IgM isotype-specific antibody capture immunoassay. With this technique, CSF JEV IgM antibodies are detectable in 70-80% of cases at the time of hospital admission, and in 100% of cases within 7 days (3-5). We prepared a simplified enzyme-linked immunosorbent assay kit for detection of JEV IgM, and stationed a technician at a provincial hospital in an endemic region to evaluate its on-site utility.

OBJECTIVE: Evaluate the utility of the JE MAC ELISA kit under field conditions.

PROGRESS: Serum and cerebrospinal fluid (CSF) obtained from patients in rural Thailand during an encephalitis epidemic were assayed with a Japanese encephalitis rapid diagnosis kit. Japanese encephalitis was diagnosed by detection of virus-specific IgM (JEV IgM) in undilute CSF or a 1:100 dilution of serum with an antibody capture enzyme-linked immunosorbent assay. Specimens were assayed immediately on-site at the provincial hospital and scored by visual examination. Each specimen was later carefully retested to accurately determine its activity (units) at a single screening dilution, and also tested at serial dilutions to determine its end-point titer. On-site kit results showed close agreement with subsequent laboratory results for detection and quantitation of JEV IgM and JEV IgG in either serum or CSF. Using the kit on-site, admission CSF from 35 (73%) of 48 laboratory-proven JEV-infected patients were scored as positive for JEV IgM, while all 17 CSF specimens from non-JEV infected patients were read as negative. A rapid and early diagnosis of acute Japanese encephalitis can be accomplished almost anywhere.

For purposes of epidemiologic surveillance, Japanese encephalitis can be diagnosed by detection of JEV IgM in dried blood eluted from filter paper strips mailed to a central laboratory (7). However, diagnostic results must be available to the physician rapidly and early in the illness if improved clinical management is to be achieved. In this study we used a simplified kit to provide early, rapid, and accurate diagnostic information to physicians in a provincial hospital in rural Thailand. We believe that this approach could be practical almost anywhere.

RECOMMENDATION: Through the WHO, standards for this test should be made available to the Ministries of Health of all nations with a known or suspected Japaneses encephalitis problem. In additions, training programs for laboratory personnel from these countries should be established under WHO auspices at AFRIMS. The Department of Virology will be providing this training in the next year.

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Viral Hepatitis Outbreaks in Thailand

BACKGROND: In a long standing arrangement with the Ministry of Public Health Training program in epidemiology, the Department of Virology continues to collaborate with trainees investigating outbreaks of viral hepatitis. Over the past several years, a number of outbreaks have been investigated, and all except two have proved to be exclusively due to hepatitis A. During the present year several outbreaks have been investigated.

OBJECTIVE: The objective of these studies is to assess the risk of various of the agents of hepatitis in epidemic hepatitis in Thailand.

PROGRESS: Summarized in the table are the outbreaks investigated to date.

HEPATITIS OUTBREAKS IN THAILAND 1982-1984

Location	Date	EIT	Etiology	No. cases	Age range	Implicated Source
Nakorn Sawan	11/82	Supramit	A ?NANB	79	10-19	Water Supply
Chiangrai	8/83	Surachai	A	61	11-30	?
Pitsanulok	11/83	Kanchanasak	A	51		Water Supply
Chaingrai	5/84	Witaya	A	36		?
Rayong	6/84	Supat	A	54	3-30	? Icecream
Phayao	7/84	Yongut	A ?NANB	62		? Water

These outbreaks illustrate several points about outbreaks of epdemic hepatitis in Thailand. 1. Epidemics occur primarily in young adults, not children. 2. Most outbreaks are due to hepatitis A. 3. NANB is not a prominant cause of epidemic hepatitis at this time.

These results are of interest because it had been presumed that young adults would be immune to hepatitis A, and therefore not susceptible to epidemics. Perhaps due to the changing standards of living in Thailand, it may be the increasing numbers of persons are reaching adulthood with out exposure. If cases in adults are more likely to be symptomatic that those in children, then increased numbers of cases in adults might be expected.

Second, the absence of significant NANB outbreaks despite recent outbreaks in Burma, Nepal, India, and Afganistan suggests that the virus(es) has not spread to Thailand. In two of the investigated epidemics, however, a number of bona fide cases appeared to be neither A nor B. Stool specimens from these cases are currently being evaluated for the presence of NANB virus. Preliminary rhesus and cynomologus challenge experiments have failed to produce serological evidence of infection, although suggestive results have been obtained by immuneelectron microscopy.

RECOMMENDATIONS:

1. Full support should be given to the epidemiology training program as they have no other source of hepatitis serology and can carry out valuable field investigations.

2. Vigorous efforts to detect outbreaks of waterborne NANB hepatitis should be continued.

3. Efforts to improve the surveillance capabilities of the Ministry of Health so that the etiology of hepatitis could be established are urgently needed.

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PROJECT 3M162770A871

PREVENTION OF MILITARY DISEASE HAZARDS

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OB 6531
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: *DD-DR&E(AR) 636*

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISS'N INSTR'N: CX
9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	AI	154 WWGH
c. CONTRIBUTING				
c.	STOG 82/83-6.2/3			

11. TITLE *(Precede with Security Classification Code)*
(U) Prevention and Treatment of Plague

12. SUBJECT AREAS
0613 Microbiology 0603 Biology

13. START DATE: 73 07
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	1.0	318
85	1.0	240

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)*
Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER *(include area code)*
202-576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
Div of CD&I
b. ADDRESS
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
WILLIAMS, J E
d. TELEPHONE NUMBER *(include area code)*
301-427-5176 or 5110
f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
HARRISON, D N
g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) RAM I; (U) Rats; (U) Mice; (U) Lab Animals; (U) Yersinia pestis; (U) Plague; (U) Diagnosis; (U) ELISA; (U) Serology; (U) Immunization

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Develop capabilities to diagnose, prevent, treat and control plague to protect troops against pneumonic and bubonic disease.

24. (U) Rapid diagnostic tests are developed and evaluated using clinical specimens. New plague vaccines and therapeutic drugs are assayed for efficacy in animal models. Strains of Y. pestis are examined for virulence and antibiotic susceptibilities.

25. (U) 83 10 - 84 09 A new enzyme immunoassay, based on monoclonal antibody, was developed for the measurement of antibodies to plague in human and animal sera. This ELISA has effectively removed the problems with nonspecificity encountered in the assay that employed sensitization of ELISA plates with F1 antigen. The new assay for antibody is similar to that now used to detect antigen, thus test protocols and design of the ELISA field kit for plague have been simplified. The new ELISA was evaluated using three groups of human sera obtained from serosurveys and clinical investigations conducted in Sudan, South Africa and Java, Indonesia. It was also employed to re-evaluate sera collected during 1970 in human volunteer studies of responses to vaccination with live, attenuated plague bacilli. In other work, a study of shelf-life for the ELISA field kit demonstrated that it is useful after 2 years of storage under optimal conditions, although shelf-life under field conditions is shorter due to deterioration of some reagents. Progress was achieved towards semi-preparative methods to produce pure lots of murine toxin and pesticin I antigens for the development and conduct of rapid immunoassays to detect these antigens, as well as antibodies to them. The process includes separation by molecular size using continuous flow membrane filtration, followed by purification and concentration from high-performance liquid chromatography. Studies of the micro-test plate for rapid biochemical characterization of suspect Y. pestis isolates continued and included shelf-life evaluations. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

PROJECT 3M162770A871 PREVENTION OF MILITARY DISEASE HAZARDS

WORK UNIT 154 PREVENTION AND TREATMENT OF PLAGUE

Investigators:

Principal: MAJ James E. Williams, MSC

Associates: Daniel N. Harrison, Ph.D.
SSG Guy L. Tyndal
William L. Roelke, Jr., MS
Sp/4 Vincent D. Palmer

Problems and Objectives

The objective is to improve capabilities for protecting troops against pneumonic and bubonic plague. Plague remains a threat to unvaccinated and possibly to vaccinated military and civilian populations in many parts of the world, especially in catastrophic or wartime situations. The potentials for plague include variant strains that are resistant to therapeutic drugs and atypical organisms that are not detected by the serological tests now used for diagnosis. The current research program focuses on three requirements for reducing the impact of plague on soldiers in combat: 1) rapid and sensitive procedures for use in the field to diagnose plague infections, to evaluate drug susceptibilities for effective therapy, and to characterize new strains of the pathogen for military intelligence, 2) improved techniques for the production and assay of plague vaccine to insure that sufficient supplies of efficacious vaccine could be prepared quickly during emergencies, and 3) a forecasting system to facilitate determinations of where and when plague surveillance or control activities may be necessary.

Progress

A new enzyme immunoassay, based on monoclonal antibody, was developed for the measurement of antibodies to plague in human and animal sera. Sera from patients with pseudotuberculosis and enterocolitis occasionally gave nonspecific reactions in the assay previously employed in this laboratory, which utilized ELISA plates sensitized with F1 antigen. The new ELISA has eliminated such problems by employing plates sensitized with anti-F1 mouse monoclonal antibody. The monoclonal antibody captures F1 antigen from a solution added to the plate. The monoclonal antibody-antigen complex subsequently captures specific F1 antibody, if present, from sera under test. Although unsuitable for testing mouse sera, this new assay is useful for evaluating sera from other sources, including rat sera. This procedure for detecting antibody is similar to the ELISA used to detect antigen, so test protocols and design of the ELISA field kit for plague have become simplified; the earlier ELISA with Fl-sensitized plates is now replaced. The new ELISA was evaluated using three groups of human sera obtained from serosurveys and clinical investigations conducted in Sudan, South Africa and Java, Indonesia. The data demonstrated the ability of the new test to define populations or areas in which plague outbreaks have and have not occurred. It was also employed to re-evaluate sera collected during 1970 in human volunteer studies of responses to vaccination with live, attenuated plague bacilli. Results were more consistent and reproducible than those obtained previously with the passive haemagglutination test. In other work, a study of shelf-life for the ELISA field kit demonstrated that it remains useful for at least two years if stored under optimal conditions. Shelf-life under field conditions is shorter due to the deterioration of some reagents. Progress was achieved towards semi-preparative methods to produce pure lots of murine toxin and pesticin I antigens of *Y. pestis* for the development and conduct of rapid immunoassays to detect these antigens, as well as antibodies to them. The process includes separation by molecular size using continuous flow membrane filtration followed by purification and concentration from high-performance liquid chromatography. In addition, candidate monoclonal antibodies to murine toxin and pesticin I have been produced. Studies of the micro-test plate for rapid biochemical characterization of suspect *Y. pestis* isolates continued with shelf-life evaluations, and results of a series of tests on enteric bacteria demonstrated that the system will rapidly separate such organisms from *Y. pestis*.

Recommendations

Continue the development of rapid immunoassays capable of detecting variant plague bacilli for use in diagnosing infections with such organisms. These new tests should target those specific antigens of *Y. pestis* that occur on plasmids which can be transferred to other bacteria, with altered virulence or disease syndrome possible. Refinements of exisitng ELISA procedures to reduce the time required for testing, to improve field portability and to remove residual "background" coloration for increased sensitivity are desirable and feasible. Development of a portable kit to rapidly determine drug susceptibilities of *Y. pestis* should be emphasized, as commercial systems currently available for this purpose do not function well for *Y. pestis*. Evaluations of new anti-bacterial drugs for chemotherapeutic value against plague should be undertaken.

Publications

1. Williams, J.E. Mise en garde contre un nouveau risque potentiel d'infection de laboratoire lie' à une nouvelle de' nomination du bacille de la peste. *Bulletin of the World Health Organization* 61(3): 547-548 (1983).

2. Williams, J.E. Riesgo de infeccion por la nueva nomenclature de bacilo de la peste. *Boletin de la Oficina Sanitaria Panamericana* 95(5): 469-471 (1983).

3. Williams, J.E. Warning: Warning on a new potential for laboratory-acquired infections as a result of the new nomenclature for the plague bacillus. *Japanese Journal of Medical Science and Biology* 36(5): 295-297 (1983).

4. Williams, J.E. Proposal to reject the new combination *Yersinia pseudotuberculosis* subsp. *pestis* for violation of the first principle of the International Code of Nomenclature of Bacteria. Request for an Opinion. *International Journal of Systematic Bacteriology* 34(2): 268-269 (1984).

5. Williams, J.E., Gentry, M.K., Braden, C.A., Leister, F. and Yolken, R.H. Use of an enzyme-linked immunosorbent assay to measure antigenaemia during acute plague. *Bulletin of the World Health Organization* 62(3): 463-466 (1984).

6. Williams, J.E. and Cavanaugh, D.C. Potential for rat plague from nonencapsulated variants of the plague bacillus (*Yersinia pestis*). *Experientia* 40: 739-740 (1984).

7. Wright, J.D., Hastriter, M.W. and Robinson, D.M. Observations on the ultrastructure and distribution of *Rickettsia tsutsugamushi* in naturally infected *Leptotrombidium (Leptotrombidium) arenicola* (Acari:Trombiculidae). *Journal of Medical Entomology* 21(1): 17-27 (1984).

PROJECT 3S162772A874

CARE OF COMBAT CASUALTY

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA 0G 6770	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62772A	3S162772A874	AG	181 WWL4
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 82/83-6.2/4			

11. TITLE (Precede with Security Classification Code)
(U) Management of Military Blast Injury

12. SUBJECT AREAS
0605 Clinical Medicine 0616 Physiology

13. START DATE 80 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	239
85	2.0	261

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER (include area code)
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME
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b. ADDRESS
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Washington, DC 20307-5100
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d. TELEPHONE NUMBER (include area code)
(202)-576-3791
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
SAMPSON, J
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
GRAEBER, G

21. GENERAL USE FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Blast injury; (U) Pulmonary dysfunction; (U) Gastrointestinal hemorrhaging; (U) Pulmonary hemorrhaging; (U) Medical/Surgical Treatment; (U) Lab animals (U) RAM II; (U) Sheep; (U) Pigs;

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23(U) This project proposes to respond to the threat of potential blast related problems which may be experienced by the Army in the field. Our ultimate goal is to optimize the treatment of blast-injured casualties. Toward this goal we are evaluating techniques which will allow documentation of the natural history of gastrointestinal and pulmonary injuries. The threat of exposure of American soldiers to blast waves from enemy weapon systems which may exceed established thresholds is increasing.

24(U) This work unit is directed at supporting field studies and special projects in the blast program. These projects involve either care of combat casualties exposed to blast waves or the pathophysiology of gastrointestinal blast injury. Animal models of blast injury are used to predict the risk that troops would encounter in various environments, and eventually to plan management including triage, evacuation, and treatment.

25(U) 83/10 - 84/09 A threat document (classified) was prepared to aid us in directing our projects at areas of most importance to the US Army and our troops at risk of injury. A major field study was carried out in conjunction with the Ballistics Research Laboratory at Aberdeen Proving Grounds to assess the blast environment within armored personnel carriers penetrated by HEAT rounds. The M2 was compared with the M113. A variety of HEAT rounds were fired. Our results will be included in the final BRL report. For technical reports see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83 - 30 Sep 84.

Project: 3S162772A874 CARE OF COMBAT CASUALTY

Work Unit: 181 Management of Military Blast Injury

Investigators:
Principal: John W. Harmon, LTC, MC
Co-Investigators: Eugene Schweitzer, CPT, MC
Barbara Bass, CPT, MC
John A. Sampson, CPT, MC
Graeber, Geoffery, LTC, MC

Background and Objectives:

The human body is vulnerable to blast overpressure. Blastwaves with significant overpressure can be created either accidentally or in a hostile battlefield environment. Very little is known about the diagnosis and care of blast injured casualties primarily because, in general, the magnitude of the blast overpressure is unknown and the injuries, which could range from mild to severe, are primarily internal. The object of the current effort is to evaluate battlefield airblast threats. The battlefield airblast threat is quite significant due to the number and variety of weapons, both conventional and nuclear, for which blast overpressure is a primary kill mechanism. The blast environment to which an unprotected soldier is subjected, of course, depends upon both the yield or energy release of the weapon as well as the standoff or range at which the soldier is located from the center of the explosion.

The first objective of this work unit was to develop a Threat Document for blast injury for soldiers in combat so as to help as target our research to benefit the injured soldier. Our second objective was to direct and participate in a field study of the effects of blast behind armor at the Aberdeen Proving Grounds in cooperation with the Ballistics Research Laboratory.

Progress:

1. Threat Definition. During the prior fiscal year a generic type threat definition for blast overpressure injury was prepared under contract by the S-Cubed group in San Diego. This report was described in last year's annual report. During this year the intelligence unit of the USAMRDC prepared a threat document with description of specific threats, documenting the requirement for studies of blast overpressure. This document is classified.

2. APG Field Study. In a letter 19 September 1983 LTC Robert L. Moore of DARCOM requested that MG Garrison Rapmund direct the USAMRDC to participate in the evaluation of blast effects behind penetrated aluminum armor. That letter referred to preliminary discussions between LTC John Harmon and the BRL staff. This project was assigned to the WRAIR under the direction of LTC Harmon, with the assistance of MAJ Yancy Phillips. Initially a protocol was developed. This protocol described animal studies to be incorporated into the BRL testig protocol to test biologically the effects of blast. The BRL controlled the decisions regarding threat weapons to be fired, number of firings, order of firing, etc. Care was taken to assure humane use of animals. LTC James Moe and his staff in the Division of Pathology planned and carried out the necropsies. The tests were carried out in the spring of 1984. Division of Surgery personnel directed the field studies in the field. This required that one officer and four enlisted be present at the test site each test day as well as on days when protocols were being evaluated. Approximatly 2500 man hours of field work were expended on this project (50 days x 5 people x 10 hours/day equals 2500). A full report of the results of the testing is being prepared.

Recommendations for the future:

A full analysis of the results of this year's test must be completed, and the full report must be prepared. Already concerns have been expressed within DOD that the BRL protocol did not answer all the possible relevant questions. We expect that a further series of tests will be required in the current year to follow-up on last year's test. This project allows the AMEDD to become familiar with the types of injuries our soldiers may sustain behind armor so it is invaluable as a means of identifying future problems in combat casualty management.

AVAILABLE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA OG 6769	84 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62772A	3S162772A874	BB	182 WWL5
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/4			

11. TITLE (Precede with Security Classification Code)
(U) Biomedical Aspects of Medical Material

12. SUBJECT AREAS
0611 Life Support 0602 Bioengineering

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER				
c. TYPE	d. AMOUNT	84	1.0	91
e. KIND OF AWARD	f. CUM/TOTAL	85	2.0	84

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Division of Surgery Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR HARMON, J W
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-3791
21. GENERAL USE FINA	f. NAME OF ASSOCIATE INVESTIGATOR (if available) KINNEY, R
MILITARY/CIVILIAN APPLICATION: H	g. NAME OF ASSOCIATE INVESTIGATOR (if available) SCHWEITZER, E COUSAR D

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Laboratory animals; (U) Medical Material Systems; (U) Biomedical support; (U) Life support systems; (U) Mice; (U) Rats; (U) RAM II

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23 (U) The primary objective is to develop and provide laboratory models for biomedical assessment of medical materiel systems. Medical materiel systems currently being developed will continue to undergo operational testing to determine if such systems are usable and useful. We will also exploit newly developed products for possible use in combat casualty management.

24 (U) Appropriate animal models and bench models will be developed and utilized to accomplish our objectives. Each medical material system will have an individual evaluation to determine which method of assessment will be used. After completion of each assessment, the data will be analyzed statistically, where possible, and a summary statement issued.

83 10 - 84 09

25 (U) In the prior year initial testing of the field auto transfusion device was completed. A report is now being prepared. Two agents were evaluated for effectiveness in treating peptic esophagitis. These were sucralfate and traysylol. A project was carried out to assess the usefulness of the clinical mass spectrometer in diagnosing ischaemic bowel as occurs in combat casualties with vascular injury. Also a project was begun to further assess the effectiveness of opiate antagonists in maintaining neurological function. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83 - 30 Sep 84.

DD FORM 1498 83 MAR

EDITION OF MAR 68 IS OBSOLETE.

AVAILABLE

Project 3S162772A874 CARE OF COMBAT CASUALTY

Work Unit 182 Biomedical Aspects of Medical Material

Investigator:
Principal: John W. Harmon, LTC, MC
Co-Investigators: Eugene Schweitzer, CPT, MC
Barbara Bass, CPT, MC

Background and Objectives:

The primary objectives are to develop and provide laboratory models for biomedical assessment of medical materiel systems. Appropriate animal models and bench models will be developed and utilized to accomplish this goal. Each medical materiel system will have an individual evaluation to determine which method of assessment will be used. After completion of each assessment, the data will be analyzed statistically, where possible, and a summary statement issued. These studies will not only assure the military relevancy of such materiels, but they will also assist in the integration of such materiels into the armamentarium of the Army Medical Corps.

Progress:

The first project was an assessment of the clinical mass spectrometer as an instrument to identify non-viable intestine at surgery. A reliable objective method to determine small bowel ischemia intraoperatively has not been developed. These experiments examined the relationship between intraluminal PCO_2 (IL pCO_2), intestinal blood flow (BF), and degree of ischemic mucosal injury. IL pCO_2 was measured with a clinical mass spectrometer using teflon catheters calibrated in tissue mode; transmural intestinal BF was measured with radioactive microspheres. Anesthetized rabbits (n=24) were cannulated for microsphere injections and mass spectrometer catheters were placed in the lumen of the small bowel. BF was determined prior to superior mesenteric artery (SMA) occlusion and then at 30, 60, or 180 minutes after occlusion. In control animals the SMA was not clamped. Intestinal biopsies were taken at the time of each BF determination and microscopic injury was graded from 1 (normal) to 4 (completeepithelial slough). Results for IL p CO_2 (mmHg) and transmural intestinal BF (ml/min·100 gm tissue) are shown here (mean ± SEM):

	SMA Occluded		Control	
	pCo_2	BF	pCO_2	BF
Baseline	89 ± 6	75 ± 9	92 ± 5	57 ± 5
30'	246 ± 26	1 ± 1	92 ± 8	46 ± 8
60'	290 ± 29	3 ± 1	93 ± 7	50 ± 8
180'	507 ± 41	1 ± 1	78 ± 9	28 ± 3

There was a strong linear correlation between the IL pCO_2 and the histologic grade of injury ($r = .778$, $p <.001$). These results show that intestinal ischemia due to SMA occlusion causes a rapid, sustained rise in small bowel IL pCO_2 tha correlates with the degree of mucosal injury. These experiments suggest that this technology may provide a superior method to assess intestinal perfusion. A report of this project has been accepted for presentation and publication by the Association for Academic Surgery, Annual Meeting.

Another project was the evaluation of sucralfate, a new anti-ulcer drug. This could potentially be useful in treating the gastric stress ulcers seen in combat casualties. We evaluated it in our rabbit esophageal model for peptic mucosal injury. Sucralfate was tested for its ability to prevent esophagitis in the perfused rabbit model. During a one hour "exposure" period the esophagus was exposed to a damaging agent (pepsin 1.0 mg/ml at pH 2, taurocholic acid 5 mM at pH 2, or trypsin 8,000 IU/ml at pH 7) or the same agents plus sucralfate (one gram per 50 ml). Then the esophagus was perfused for a one hour "flux" period to assess mucosal permeability to sodium, potassium, glucose, and ^{14}C-erythritol. Finally, the animals were sacrificed and esophageal specimens were graded in a blind fashion for gross esophagitis using a point system (1 = normal to 4 = most severe injury). The results of the acid-pepsin experiments are shown below (six animals per group):

	Erythritol umoles/min	Na uEq/min	K uEq/min	Glucose ugm/min	Esophagitis Index
Pepsin free	-1 ± 3	1 ± 1	0.1 ± 0.1	5 ± 5	1.7 ± 0.2
Pepsin	-23 ± 6	6 ± 1	0.4 ± 0.1	71 ± 13	3.5 ± 0.2
Pepsin & Sucralfate	-3 ± 5	1 ± 1*	0.1 ± 0.1*	12 ± 3*	1.7 ± 0.2*

*Significantly less ($p< 0.05$) than pepsin alone

In contract to the results above showing that sucralfate can prevent the damaging effects of pepsin, it provided no signficant potection from the actions of trypsin or bile acid. Adherence of the sucralfate to the mucosa was demonstrated in the pepsin experiments by ultraviolet fluorescence after quinine staining. This was probably a critical factor in its activity since in vitro testing revealed inactivation of less than 20% of the pepsin in solution. As our other experimental work suggests that pepsin is the primary damaging agent in acid reflux esophagitis, sucralfate may be useful therapeutic agent for this condition.

The Division of Surgery is also working to develop an autotransfusion device suitable for forward field use. In the past year testing of this device was carried out. Red cell survival and clotting function were tested after the blood from a simulated war wound passed through the device. The data from these experiments is still being analyzed.

Recommendations for the Future:

This project responds to needs as they arise. Currently Traysylol, a trypsin inhibitor is being evaluated as an anti ulcer drug. Other agents are on the horizon. A decision will be required as to the potential clinical usefulness of the Field Autotransfusion devices. RAM II USAMRDC is determining whether a requirement for this device exists. Meanwhile a patent application is pending. The Division will soon be field testing an air droppable field hospital of French design. This will be a major project of the next fiscal year. Another project for next year is the evaluation of Naloxone as an agent to prevent the paraplegia which occasionally results from surgical cross clamping of the abdominal or thoracic aorta.

PROJECT 3M162734A875

MEDICAL DEFENSE AGAINST CHEMICAL AGENTS

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OC 6479	2. DATE OF SUMMARY 841001	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 831001	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62734A	3M162734A875	AJ	161 WWME
b. CONTRIBUTING				
c. XXXXXXXXXX	STOG 82/83-6.2/1			

11. TITLE *(Precede with Security Classification Code)*
(U) Development of Anti-Chemical Warfare Drugs

12. SUBJECT AREAS
0603 Biology 0615 Pharmacology 0703 Organic Chemistry

13. START DATE 7810	14. ESTIMATED COMPLETION DATE Cont	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	5.0	482
85	5.0	585

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)*
Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H Jr
d. TELEPHONE NUMBER *(include area code)*
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
Division of Experimental Therapeutics
b. ADDRESS
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
DAVIDSON, D E
d. TELEPHONE NUMBER *(include area code)*
(301)-427-5411
f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

21. GENERAL USE FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U)Drug Development; (U)Chemical Defense; (U)Molecular Modeling; (U)Chemical Poisons; (U)Nerve Agents; (U)Chemical Synthesis; (U)RAM V

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23.(U) To develop new drugs with protective activity against injury to military personnel in the event of exposure to chemical poisons.

24.(U) Potentially active drugs will be identified and obtained by synthesis or purchased. Candidate drugs will be tested in laboratory model systems to establish protective efficacy, mechanisms of pharmacological effects, effects on physiological response and pharmacokinetic characteristics. Results will be used as input to computer-assisted molecular modeling system for evaluation to guide design of new compounds. Information is used in selection of candidate drugs for clinical trials.

25.(U) 8310-8409 In the primary screen for efficacy against toxic doses of cyanide in mice, more than 300 compounds were tested. Eight compounds with protective activity of at least 70% were studied for further definition of efficacious dose and for activity in combination with sodium thiosulfate. In therapeutic studies, a mixture of sodium cobaltinitrite and sodium nitrite was ineffective as an antidote for KCN at 10 mg/kg. Chlorpromazine increased the protective effect of sodium thiosulfate and of the combination of sodium thiosulfate and sodium nitrite. Highest Protective Index obtained therapeutically was 9.16, by a combination of sodium thiosulfate, sodium nitrite and hydroxocobalamine. Substitution of dimethylaminophenol for sodium nitrite gave a comparable Protective Index (8.21). Four extramural testing systems are supported by contract. Synthesis of prospective compounds is supported by an extramural program of 25 contracts under which compounds are synthesized as oxime reactivators of acetylcholinesterase, non-oxime reactivators, antimuscarinic and pretreatment drugs. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83-30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

PROJECT: 3M162734A875 MEDICAL DEFENSE AGAINST CHEMICAL AGENTS

WORK UNIT: 161 Development of Anti-Chemical Warfare Drugs

INVESTIGATORS:

Principal: COL David E. Davidson, Jr., VC

Associate: LTC William H. Amos, Jr., MS
Dr. Melvin H. Heiffer
Dr. Gerald J. McCormick
LTC Jonathan D. Berman
LTC William E. Ridder, VC
Dr. David Davis
Dr. Hikmat A. Musallam
Ms Marie M. Grenan
Dr. A.J. Lin
Dr. Lily C. Tang

PROBLEM AND OBJECTIVES:

Antidotes currently available to protect or treat U.S. military personnel who may be attacked with chemical weapons are inadequate and, for some types of chemicals which are potential threats, antidotes are non-existent or unsuitable for mass administration. The development of suitable antidotes and pretreatment drugs would improve the capacity of military units to perform effectively in the event of use of chemical agents, reduce the number of medical casualties and deter the use of chemical agents by an enemy.

PROGRESS:

Intra- and extramural programs of synthesis and testing of candidate compounds have been conducted.

The extramural synthesis program is supported by 25 contracts under which compounds are synthesized as oxime reactivators of acetylcholinesterase, non-oxime reactivators, antimuscarinic and pretreatment drugs. 250 compounds were submitted for testing from the synthesis program and, in addition, there are contracts for radiolabeling, larger scale preparations, chemical analyses and data handling and inventory services. As an adjunct to the extramural program, intramural studies of stability of reactivators are in progress. Three compounds have been examined, MMB-4, TMB-4 and HI-6, and of these, TMB-4 appears to be the most stable. Several decomposition products were isolated by preparative thin-layer chromatography and identified by nuclear magnetic resonance and gas chromatography/mass spectrometry analytical procedures.

In the intramural primary screen for efficacy by intraperitoneal administration against toxic doses of cyanide in mice, more than 300 compounds were tested. Eight compounds, including alkylaminothiol derivatives (simple thiols, dithiols, disulfides, phosphorothionates and sulfonates), with protective activity of at least 70% were studied for further definition of efficacy and toxicity. Studies included determinations of activity in combination with sodium thiosulfate, duration of effectiveness and activity after oral administration. In the intramural therapeutic study program, combinations of drugs have been examined for efficacy against cyanide in mice. A mixture containing equimolar sodium cobaltinitrite and sodium nitrite was ineffective as an antidote for KCN at 10 mg/kg. Chlorpromazine by prior administration increased the protective effect of sodium thiosulfate (protective index (PI) increased from 1.25 to 3.1) and the effect of the combination of sodium thiosulfate (200 mg/kg) and sodium nitrite (50 mg/kg), increasing the protective index from 3.68 to 4.97. The combination of chlorpromazine and dimethylaminophenol had no more efficacy than dimethylaminophenol alone. Dimethylaminophenol potentiated the effect of sodium thiosulfate at 200 mg/kg, increasing the protective index from 1.25 to 5.65. The highest protective index obtained was 9.16 (range 8.3 to 10.1), achieved by the combination of sodium thiosulfate (500 mg/kg), sodium nitrite (50 mg/kg) and hydroxocobalamine (1000 mg/kg). Substitution of dimethylaminophenol (30 mg/kg) for sodium nitrite achieved comparable effect (protective index of 8.21, range 7.42 to 9.09).

An intramural program of molecular modeling is in process of acquiring computer hardware and software.

FUTURE OBJECTIVES:

Directed synthesis of compounds in chemical classes with known or hypothesized activity as protectants against chemical agents will continue. New synthetic routes will be developed to allow greater recovery of compounds and preparation of novel chemicals of interest. Laboratory models currently in use or under development will be employed in testing the efficacy of candidate compounds and in the investigation of modes of activity. Laboratory facilities for intramural study of cholinergic muscarinic agonists including organophosphates will be completed. Computer capability will be developed to include molecular modeling and biological data systems.

VAILABLE

PUBLICATIONS:

1. Tang, L.C., Schoomaker, E., and Weismann, W.P. 1984. Cholinergic Agonists Stimulate Calcium Uptake and cGMP Formation in Human Erythrocytes. Biochemica et Biophysica Acta 772, 235-238.

2. Tang, L.C. 1984. A Personal and Scientific Biography of Dr. George C. Cotzias. Neuro Toxicology 5, 5-12.

PRESENTATIONS:

1. Davidson, D.E., Davis, D., and Canfield, C.J. 1984. Chemoprophylaxis of Cyanide Intoxication. Fourth Annual Chemical Defense Bioscience Review, Aberdeen, MD. June, 1984.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION DA 305977	2. DATE OF SUMMARY 84 10 01	*REPORT CONTROL SYMBOL.* DD-DR&E(AR) 636
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX
9. LEVEL OF SUM A. WORK UNIT					

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY	62734A	3M162734A875	AL	162	WWJK
b. CONTRIBUTING					
c. CONTRIBUTING	STOG 82/83-6.2/1				

11. TITLE *(Precede with Security Classification Code)*
(U) Effects of therapeutic and prophylactic drugs on human performance

12. SUBJECT AREAS
0510 Psychology, 0616 Physiology, 0615 Pharmacology, 0605 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
83 10	CONT	DA	C. In-House

17. CONTRACT GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	1.2	92
85	2.0	179

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Neuropsychiatry
b. ADDRESS *(include zip code)* Washington, D.C. 20307-5100	b. ADDRESS Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR O'DONNELL, V M
d. TELEPHONE NUMBER *(include area code)* 202 - 576-3551	d. TELEPHONE NUMBER *(include area code)* 301 - 427-5521
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* HEGGE, F W g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* REDMOND, D P

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) RAM V
(U) Drugs; (U) Chemical Defense; (U) Performance; (U) Volunteers

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The objective is to specify the military performance effects of drugs prescribed for the treatment or prevention of disease or injury, especially those related to chemical defense.

24. (U) Approach is to: 1) identify therapeutic and prophylactic drugs which have high probabilities of use during military operations and of affecting military performance; 2) identify behavioral and physical performance variables sensitive to these drugs; 3) develop laboratory and field methods for the measurement of these variables; 4) assess the effects of selected drugs on these variables; 5) validate the variables and assessment instruments as predictors of military performance through field studies and system model simulations; 6) develop models which relate the physiologic actions of the drugs to the intervening variables and military performance; 7) validate, modify, and expand these models; 8) recommend doctrine.

25. (U) 83 10 - 84 09. Work was previously reported under "Soldier Performance Capabilities Under Chemical Agent Prophylaxis and Therapeutics". Protocol for measuring the psychophysiological effects of atropine on human subjects in relation to blood levels of the drug, has been written and approved; subjects have been recruited. An automated bibliography of 3,100 references relevant to neurochemistry and performance has been compiled. A contract for the development of PERT/CPM models of weapon systems operation has been let. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 1498 83 MAR   EDITION OF MAR 68 IS OBSOLETE.

Project: 3M162734A875 MEDICAL DEFENSE AGAINST CHEMICAL AGENTS

Work Unit: 162 Effects of Therapeutic and Prophylactic Drugs on Human Performance

Investigators:

Principal: Frederick Hegge, Ph.D.

Associates: Vincent O'Donnell, Ph.D., CPT MS

Gary Harding, M.S.

Problems and Objectives:

This work unit studies the effects of chemical defense prophylactic and therapeutic drugs on soldier cognitive performance and neuro-behavioral homeostasis.

Progress:

A protocol was written and approved by WRAIR scientific review and WRAIR and SGO human use committees to study the kinetics and blood levels of atropine as they relate to changes in human cognitive performance and affective state. The micro-computer based tests to support the study have been developed and programmed for Apple Computers and are now operational. The actual running of subjects began in the last week of FY84 and will continue well into FY85.

An automated bibliographic retrieval system containing 3,100 references relevant to the neurochemical aspects of behavior has been developed and is operational. In addition, recording and keywording of an additional 300 references for the second edition of the system has been completed. Upon request, the system has been provided to investigators at LAIR, USAARL, the University of Hawaii, and Bar Ilan University. RAD V at USAMRDC and the Naval Medical Research Unit have requested the information system.

The literature was reviewed on pyridostigmine and performance. This review was presented to the Joint Working Group on the Drug Dependent Degradation of Military Performance.

Substantial in-house development work was done on integrating departmental non-invasive medical monitoring systems into a combined system relevant to assessing the effects of chemical weapon prophylactic and therapeutic drugs. This involved extensive computer-systems integration at both the hardware and software levels. Advances were made in developing a computer-based data processing of the data obtained with the combined system.

In preparation for future studies, the department is acquiring the capability to measure metabolic rate. The role of metabolic rate as an intervening variable in the performance effects of atropine will be studied using a micro-computer based metabolic monitor able to assess metabolic rate on a breath-by-breath basis. Data will be related to other drug studies and sleep studies to provide information on how metabolic rate affects cognition, mood, and performance. Because metabolic rate is readily affected by such factors as temperature, age, exercise, diet, circadian rhythms, and drugs, it represents a variable which will be altered by military operations. Metabolic rate, however, has never been investigated in relation to performance, although there is indirect evidence that it impacts heavily on performance. If this relationship is borne out by our studies, it will provide a point of intervention to counter the performance degrading effects of chemical defense drugs.

Cholinergic action has recently been implicated in the activation of a number of neuroactive hormones and peptides. A series of studies will be undertaken in human subjects to determine if atropine interferes with the activation of these hormones. Peripheral and central effects of atropine on these hormones will be discriminated, and the effects on specific hormones and peptides will be correlated with performance changes.

Publications: None

Presentations: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA305978	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
	A. NEW	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62734A	3M162734A875		WWHF 163
b. CONTRIBUTING				
c. XXXXXXXXXX	STOG 82/83-6.2/1			

11. TITLE (Precede with Security Classification Code)
(U) Physico-chemical Evaluation of Nerve Agent Antidotal Formulations

12. SUBJECT AREAS
0615 Pharmacology 0703 Organic Chemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	Cont	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	0.0	00
85	2.0	270

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
Top, F H Jr
d. TELEPHONE NUMBER (include area code)
(202) 576-3551

20. PERFORMING ORGANIZATION
a. NAME Division of Biochemistry
Walter Reed Army Institute of Research
b. ADDRESS
Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
Brown, N D
d. TELEPHONE NUMBER (include area code)
(202) 576-3020
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
Doctor, B P
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Nerve Agent Antidotes ; (U) Stability ; (U) Oximes , (U) Benzylates (U) RAM V

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objective of this work unit is to develop, formulate, and determine the suitability of the single and multicomponent nerve agent antidotal preparations for self-administration in the field. There is military relevance in this research.

24. (U) Analytical, Biochemical, Immunological, and Pharmacological methods will be employed to determine the bioefficacy, stability, packaging and in case of multicomponents synergisum of multicomponent formulations containing nerve agent antidotes. The effects of pH, temperature, ionic strength, antifungal agents, containers and light on the stability of antidotal components will be determined. Spectrophotometric, HPLC, AC, MS, NMR techniques will be employed to determine and elucidate the degradation product formed (if any). Pharmacokinetics, distribution, clearance, bioavailability and metabolic products identification of potentially efficacious compounds will be carried out. The antidotal compounds to be studied are oximes, benzylates, antinicotinic and antimuscarinic agents.

25. (U) None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OG 8600	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62734A	3M162734A875	AL	164 WWJF
b. CONTRIBUTING				
~~c. CONTRIBUTING~~	STOG 82/83-6.2/1			

11. TITLE (Precede with Security Classification Code)
(U) Behavioral Toxicology

12. SUBJECT AREAS
0510 Psychology 0616 Physiology 0615 Pharmacology

13. START DATE 80 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	460
85	2.0	499

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Neuropsychiatry
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Elsmore, T F
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-2483
21. GENERAL USE F I N A MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Leu, J R g. NAME OF ASSOCIATE INVESTIGATOR (if available) Witkin, J M

22. KEYWORDS (Precede EACH with Security Classification Code) (U)Chemical Defense;(U)Behavior;(U)Toxicology:(U) Neuropsychiatry;(U)Chronopharmacology;(U)Lab Animals:(U)Rats:(U)Monkeys:(U)RAM V:

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Methods for assessing the impact of chemical defense-related compounds upon behavior will be evaluated. The overall objective will be development of testing protocols with maximum sensitivity to behavioral effects of chemical defense agents for use in the Army's effort to develop and field better antidotes to chemical agent exposure.

24. (U) The techniques of operant and respondent conditioning will be used to generate behavioral baselines which will be sensitive to the effects of chemical defense-related compounds. Dose-effect and time course functions will be determined in rodents and primates on procedures spanning a range of behavioral functions. Chronopharmacological effects will be evaluated. Methods of curve fitting and time series analysis will be used to evaluate drug effects.

25. (U) 83 10 - 84 09 Major findings: No differences between effects of atropine and atropine methyl nitrate on either accuracy or work rate were found in separate studies of short-term auditory memory in rats and visual memory in monkeys. These results suggest that accuracy decrements may be due to peripheral mechanisms in these tasks. A behavioral pharmacology laboratory for studying drug effects in squirrel monkeys was established. Initial studies in this laboratory discovered a non-muscarinic neurotoxic action of the muscarinic receptor agonist oxotremorine. Disruptions in rats' performance of a spatial memory task by physostigmine, a reversible cholinesterase inhibitor, was reversed by atropine but not by mecamylamine, demonstrating that the effect was muscarinic in nature. Long-term effects of inhibition of cholinesterase by organophosphate compounds was shown to produce increases in elasticity of demand for food. For technical report see Walter Reed Army Institute of Research Annual Report, 1 Oct 83 - 30 Sep 84.

AVAILABLE

Project: 3M162734A875 MEDICAL DEFENSE AGAINST CHEMICAL AGENTS

Work Unit: 164 Behavioral Toxicology

Investigators:
Principal: Elsmore, T.F., Ph.D.
Associate: Hursh, MAJ, S.R.; Raslear, CPT, T.G.; Leu, CPT, J.R.; Witkin, CPT, J.M.; Parkinson, J.K., MA

Objectives:

The overall objective of this work unit is the development of testing methods to evaluate behavioral effects of compounds used for pretreatement or therapy of chemical warfare agent exposure. This work unit will evaluate both the inherent toxicity of these compounds and their efficacy in preventing possible long-term behavioral and neurological deficits produced by these agents.

Progress:

There have been two major continuing efforts in this area in the past year, studies on the behavioral toxicology of atropine and other cholinergic compounds, and studies on long-term sequelae of organophosphate (OP) poisoning.

A major goal of this work unit is the development of rapid, inexpensive assays for behavioral toxicity. Further, it is desirable that these assays provide some information regarding the specific behavioral functions affected. We have continued to evaluate the effects of a variety of cholinergic agents on performance of rats in the radial arm maze. The muscarinic agonist compound physostigmine was shown to produce decrements in performance of this task which were reversed by the muscarinic receptor antagonist atropine sulfate but not by the nicotinic receptor antagonist mecamylamine, suggesting that the effects of physostigmine are solely on muscarinic receptors.

Another goal of this work unit is the demonstration of generality of results across species. We previously reported that the central and peripheral actions of atropine could be separated in a spatial memory task in rats. Similar results have now been found in a learning task with monkeys who were required to repeatedly learn new sequences of responding to obtain food. Atropine sulfate and atropine methyl nitrate both decreased the rate at which the problems were solved, but only atropine sulfate increased errors during the solution process. Attempts to generalize this finding to a short-term memory task in both rats and monkeys have been unsuccessful. Both compounds produce equivalent deficits in accuracy.

The environment in which an individual is exposed to cholinergic agents may also determine the effect of those agents on performance. Several drugs have been shown to differentially affect performances of squirrel monkeys that are maintained by different events. For example, the anti-anxiety drug chloriazepoxide increases responding maintained by food presentation but only decreases responding maintained by shock. In this situation we found that the effects of atropine could be qualitatively altered by manipulating the time between drug injection and behavioral testing; atropine only decreases responding when given immediately prior to experimental sessions but can produce two-fold increases in responding when given two hours prior. These time-dependent excitatory effects can also be seen with scopolamine suggesting that such behavioral changes may be a general feature of the occupation of muscarinic receptors by antagonists. A series of studies is well under way to evaluate the role of central muscarinic receptors in the effects of physostigmine. Physostigmine produces dose-dependent decreases in responding with concomitant increases in the duration of responses. Neostigmine, a quaternary analog of physostigmine which only poorly penetrates into the central nervous system, produces similar deteriorations in behavior within the same range of doses. Although all of the peripheral actions of physostigmine are also reversed by methylatropine, comparable effects of physostigmine on behavior, however, are not reversed by methylatropine. Behavioral effects of physostigmine are, however, completely antagonized by the centrally-acting muscarinic antagonist atropine even when atropine is given in doses which decrease avoidance behavior in their own right. The results of these studies thus far indicate that cholinesterase inhibitors like physostigmine produce potent decrements in behavioral performances which appear to be due primarily to the activation of muscarinic receptors in the central nervous system.

Another major area of research involves studies of the non-cholinergic actions of cholinergic compounds. The aim of this research is to determine the nature and extent of the reversibility or prevention of the behavioral toxicity of cholinergic drugs by non-cholinergic agents. Although the major thrust of this research is yet to be initiated, we have recently determined that, in squirrel monkeys, the muscarinic receptor agonist, oxotremorine, in high doses, produces a delayed toxicity which may be non-muscarinic in nature, since it is not prevented by treatment with high doses of atropine.
We have previously reported that the CW agent soman produces brain lesions in rats following a single dose. The cholinesterase inhibitor DFP also produces disruptions in circadian feeding and activity patterns. In addition, there are numerous reports in the literature of long-term psychiatric and neurological changes in humans following accidental exposure to OP compounds. Several studies are being performed in rats to define the behavioral deficits that may be expected following organophosphate poisoning, and to determine the effects of several standard therapeutic drugs in preventing these long-term effects. This year we showed that while the level of ad libitum food intake does not

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change following a single dose of DFP the slope of the demand curve for food increases. That is, motivation to obtain food decreases. These data were gathered using a simple technique in which the amount of work required to obtain food is increased by a small amount each day. This technique has great promise as a cost-effective assay for enduring effects of exposure to CW agents, and the efficacy of pretreatment and therapeutic compounds in prevention of these effects. Work in this area will be significantly aided by the establishment of a facility within the WRAIR for the use of dilute agents. This facility is expected to be available early in FY 85.

Future objectives:

The primary focus will continue to be the development of cost-effective techniques for evaluating behavioral effects of CW pretreatement and therapeutic compounds. Studies evaluating environmental determinants of behavioral effects in monkeys will continue. Efforts to partition central and peripheral effects of atropine will continue. Disruption in estrus cycling of female rats by exposure to OP compounds and antidotes will begin. Findings made with the agent simulator DFP will be replicated with soman.

Presentations

Elsmore, T.F. (1984) Effects of repeated administration of atropine on DMTS performance of monkeys. Association for Behavior Analysis, Nashville.

Witkin, J.M. (1984) Tolerance to the water intake-suppressant effects of chlordimeform. IUPHAR 9th International Congress of Pharmacology, London, 1121P.

Witkin, J.M. (1984) Behavioral effects of some benzodiazepine receptor ligands in the pigeon. Paper presented to the Behavioral Pharmacology Study Group, Johns Hopkins University and the NIDA Addiction Research Center, Baltimore, Maryland, April.

Publications

Barrett, J.E., Brady, L.S. and Witkin, J.M. Behavioral studies with anxiolytic drugs. I. Interactions of the benzodiazepine antagonist Ro 15-1788 (flumazepil) with chlordiazepoxide, pentobarbital and ethanol. Journal of Pharmacology and Experimental Therapeutics, in press.

Barrett, J.E. and Witkin, J.M. The role of behavioral history in determining the effects of abused drugs. In S.R. Goldberg and I. Stolerman (Eds.) Behavioral Analysis of Drug Dependence, Academic Press, in press.

Barrett, J.E., Witkin, J.M. and Mansbach, R.S. (1984) Behavioral and pharmacological analysis of the effects of buspirone. *Federation Proceedings*, **43**, 931.

Leu, John R., Raslear, Thomas G., & Luz, George A. (1984). The cholinesterase inhibitor diisopropyl phosphoroflouridate (DFP) retards habituation of acoustic startle. Presented at the annual meeting of the Eastern Psychological Association. *Proceedings and Abstracts of the annual meeting of the Eastern Psychological Association*, *55*, 28.

Leu, J. R. & Raslear, T. G. (1983). Effects of diisopropyl phosphorofluoridate on circadian activity patterns in rats. Presented at the annual meeting of the Society For Neuroscience. *Abstracts: Society for Neuroscience*, *9*(2), (Abstract # 313.9).

Levy, A., Elsmore, T.F. and Hursh, S.R. (1984) Central vs peripheral anticholinergic effects on repeated acquisition of behavioral chains. *Behavioral and Neural Biology*, **40**, 1-4.

Levy, A., Kluge, P.B., and Elsmore, T.F. (1983) Radial arm maze performance of mice: Acquisition and atropine effects. *Behavioral and Neural Biology*, **39**, 229-240.

Raslear, T.G. and Kaufman, L. (1983) Diisopropyl Phosphorofluoridate (DFP) disrupts circadian activity patterns. *Neurobehavioral Toxicology and Teratology*, **5**, 407-411.

Weissman, B.A., Barrett, J.E., Brady, L.S. Witkin, J.M., Mendelson, W.B., Paul, S.M. and Skolnick, P. (1984) Behavioral and neurochemical studies on the anticonflict actions of buspirone. *Drug Development Research*, **4**, 83-93.

Witkin, J.M. Effects of some volatile sedative-hypnotics on punished behavior. *Psychopharmacology*, in press.

Witkin, J.M., Barrett, J.E., Cook, J.M. and Larscheid, P. (1984) Differential antagonism of diazepam-induced hypnosis. *Federation Proceedings*, *43* 930.

Witkin, J.M. Dworkin, S.I. and Katz, J.L. (1984) Behavioral effects of chronic *d*-amphetamine administration. *Psychopharmacology*, **83**, 53.

Witkin, J.M., Sickle, J. and Barrett,J.E. (1984) Potentiation of the behavioral effects of pentobarbital, chlordiazepoxide and ethanol by thyrotropin-releasing hormone. *Peptides*, **5**, 809-814.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA30597 9	2. DATE OF SUMMARY 84 10 01	*REPORT CONTROL SYMBOL* *DD-DR&E(AR) 636*
3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. NEW	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62734A	3M162734A875		WWHE 165
b. CONTRIBUTING				
c. XXXXXXXXXX	STOG 82/83-6.2/1			

11. TITLE *(Precede with Security Classification Code)*
(U) Rapid evaluation of potential anticholinergic drugs

12. SUBJECT AREAS
0615 Pharmacology 0601 Biochemistry 0603 Biology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	0.0	00
85	1.0	152

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of Biochemistry Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)* Washington, D.C. 20307 - 5100	b. ADDRESS Washington, D.C. 20307 - 5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Chiang, P K
d. TELEPHONE NUMBER *(include area code)* (202) 576-3551	d. TELEPHONE NUMBER *(include area code)* (202) 576-1361
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Gordon, R K g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Doctor, B P

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Receptors; (U) Muscarinic; (U) Nicotinic; (U) Antagonists; (U) Agonists (U) RAM V

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The objective of this work unit is to evaluate potential antimuscarinic and antinicotinic compounds for their ability to: 1) block the binding of radioactive antagonists and/or agonists to their respective receptors by using various neuronal cultures; 2) block the release of amylase from pancreatic cells stimulated by carbachol in the case of antimuscarinic agents; 3) block the contraction of guinea pig ileum stimulated by acetylcholine. There is military relevance in this research.

24. (U) The receptor binding assays will be carried out by using the following lines: N4TGI neuroblastoma cells that have muscarinic receptors but not the nicotonic receptors; NG108-15 neuroblastoma x glioma hybrid cells that possess both the muscarinic and nicotinic receptors. In addition, purified muscarinic receptors will be used once purified from a suitable source. The compounds (antimuscarinic and nicotinic) synthesized under USAMRDC contracts will be tested in the primary screening tests for the anticholinergic potency. Those found effective will be recommended for further evaluation, and structures of compounds which may provide better potency will be predicted and recommended for synthesis.

25. (U) None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 300534
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62734A	3M162734A875	AK	166 WWHA
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 82/83-6.2/1			

11. TITLE (Precede with Security Classification Code)
(U) Antiradiation Drug Development

12. SUBJECT AREAS
0703 Organic Chemistry 0618 Radiobiology

13. START DATE: 82 10
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	171
85	2.0	247

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code): Washington, D.C. 20307 - 5100
c. NAME OF RESPONSIBLE INDIVIDUAL: Top, F H Jr
d. TELEPHONE NUMBER (include area code): (202)-576-3551
e. GENERAL USE: FINA
MILITARY/CIVILIAN APPLICATION: H

20. PERFORMING ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS:
Division of Biochemistry
Washington, D.C. 20307 - 5100
c. NAME OF PRINCIPAL INVESTIGATOR: Alving, C R
d. TELEPHONE NUMBER (include area code): (202)-576-3248
f. NAME OF ASSOCIATE INVESTIGATOR (if available): Richardson, E
g. NAME OF ASSOCIATE INVESTIGATOR (if available): Wassef, N

21. KEYWORDS (Precede EACH with Security Classification Code) (U) Liposomes; (U) RAMV; (U) Antiradiation Drugs
22. (U) Drug Carriers; (U) Antibody; (U) Parasites; (U) Endotoxin; (U) Arachidonic acid

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The objective is to develop liposomes as carriers of drugs and agents for protection against radiation effects involves cells of the reticuloendothelial system. Macrophages are key cells that may play a pivotal role in protection against radiation. The objective will be to determine the effectiveness of liposomes for delivery both of conventional antiradiation drugs and also of agents that stimulate macrophages to produce increased hematopoietic activity. There is military relevance in this research.

24. (U) The approach will be to use inbred mice that have been lethally or sublethally irradiated as a model to determine the effectiveness liposome-encapsulated drugs. Effectiveness will be judged both by increased survival of the animals and by increased hematopoietic activity in the spleen. A standard test of hematopoietic activity that was originally developed at WRAIR and is now widely used is the appearance of nodules that signify hematopoietic activity in the spleen. These nodules presumably are induced by the secretion of colony stimulating factor by macrophages.

25. (U) 83 10 - 84 09 Antiradiation protection induced by injection of liposomes alone was discovered. Markedly enhanced antiradiation protection was observed after injection of liposome-associated lipid A when compared to liposomes or lipid A alone. These two substances were synergistic, and this was probably due to induction of colony stimulating factor from macrophages by liposome-associated lipid A. When compared with glucan, a potent stimulator of antiradiation protection, the liposome-associated lipid A was less effective but had the same order of effectiveness. For technical report see WRAIR Annual Progress Report 1 Oct 83 - 30 Sept 84.

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PROJECT: 3M162734A875 MEDICAL DEFENSE AGAINST CHEMICAL AGENTS

WORK UNIT: 166 Antiradiation Drug Development

INVESTIGATORS:
Principal: Carl R. Alving, M.D., COL, MC
Associates: Nabila M. Wassef, Ph.D., DAC; Earl C. Richardson, DAC
Assistant: SP4 Denton Hargis, PFC Michael Stout

DESCRIPTION:

The approach was to use inbred mice that had been lethally or sublethally irradiated as a model to determine the effectiveness liposome-encapsulated drugs. Effectiveness was judged both by increased survival of the animals and by increased hematopoietic activity in the spleen. A standard test of hematopoietic activity that was originally developed at WRAIR is now widely used is the appearance of nodules that signify hematopoietic activity in the spleen. These nodules presumably are induced by the secretion of colony stimulating factor by macrophages. One agent that is known to be one of the most effective stimulators of hematopoietic activity (lipid A from endotoxin) was tested both alone and in liposomes. Using this substance enhanced hematopoietic activity was demonstrated with liposomes. In addition the liposomes alone had some enhanced hematopoietic activity, and the possibility that this was due to endotoxin contamination is being investigated.

The influence of liposomes alone and liposomes containing phosphatidylinositol was examined with macrophages. The macrophage is a key cell for stimulating antiradiation protection. The influence of lipid A in lymphocytes was also examined. The overall summary of the work, which is still at an intermediate stage, would indicate that liposomes have a substantial capability to promote radiation protection by serving as carriers of antiradiation agents.

1. Suppression of phagocytic function and phospholipid metabolism in macrophages by phosphatidylinositol liposomes

Increased phagocytosis of complement-opsonized vesicles was accompanied by increased phosphatidylinositol (PtdIns) turnover in murine macrophages. However when (PtdIns) was also present as one of the lipids in the opsonized liposomes, it reduced both phagocytosis and stimulation of endogenous PtdIns turnover. These suppressive effects did not occur with liposomes containing PtdIns phosphate (PtdIns-P). When a monoclonal IgM "anti-PtdIns-P" antibody that bound to inositol phosphate was substituted for antigalactosyl ceramide antibodies for activating complement in the opsonizing process, enhanced phagocytosis occurred normally but increased cellular PtdIns turnover did not occur. Therefore the data show that, although PtdIns-P cannot replace PtdIns for suppressing PtdIns turnover, PtdIns-P can be induced to be suppressive after specific binding to an antibody that recognizes inositol phosphate. We conclude that ingestion of complement-opsonized liposomes by macrophages and complement-induced turnover of cellular PtdIns are separate but related phenomena that can be independently modulated by the polar group of liposomal PtdIns.

2. Mitogenic Response of Lymphocytes from C3H/HeJ Mice in the Presence of Lipid and Lipid A Fractions

Purified lipid A and eight fractions of lipid A were examined for the ability to elicit a mitogenic response in spleen lymphocytes of endotoxin-resistant C3H/HeJ mice. The pattern of results obtained with different lipid A fractions was similar to that observed with spleen cells from the endotoxin-responsive C57B1/6J mouse. Although the absolute mitogenic response of C3H/HeJ lymphocytes to lipid A was diminished, the cells apparently were completely normal with respect to the pattern of relative mitogenic responses induced by purified fractions of lipid A.

3. Mitogenic Activities of Lipid A and Liposome-Associated Lipid A: Effects of Epitope Density

Among eight purified lipid A fractions, there was heterogeneity of mitogenic activity, but the relative activity could not be predicted by limulus amebocyte lysate assay. Analysis of mitogenic activity after the incorporation of lipid A into liposomes demonstrated that epitope density of lipid A was a crucial factor that strongly modulated expression of lipid A. The results suggest that lipid A hydrophobic groups are responsible for mitogenic activity and that the surface accessibility of lipid A hydrophobic groups in membranes may be influenced by epitope density. The data are compared with previous results derived from parallel studies on limulus amebocytes. Both approaches lead to the conclusion that the activities of purified lipid A fractions are heterogeneous and that epitope density is critically important for the expression of biologic activity of membrane-associated lipid A.

PUBLICATIONS

1. Alving, C.R. and E.C. Richardson (1984). Mitogenic activities of lipid A and liposome-associated lipid A. Effects of Epitope Density. Revs. Inf. Dis. 6, 493-496.

2. Richardson, E.C. and C.R. Alving (1984). Mitogenic Response of Lymphocytes from C3H/HeJ Mice in the Presence of Lipid A and Lipid A Fractions. Revs. Inf. Dis. 6, 532-534.

3. Wassef, N.M., Roerdink, F., Richardson, E.C. and C.R. Alving (1984). Suppression of Phagocytic Function and Phospholipid Metabolism in Macrophges by Phosphatidylinositol Liposomes. Proc. Natl. Acad. Sci. U.S.A. 81, 2655-2659.

PUBLISHED ABSTRACTS-PRESENTATIONS

1. Wassef, N.M. and C.R. Alving (1984). Concanavalin-A Induced Phagocytosis of Liposomes Containing Phosphatidylinositol by Murine Macrophages FASEB Annual Meeting.

2. Wassef, N.M., Roerdink, F., Richardson, E.C. and C.R. Alving (1984). Suppression of Phagocytic Function and Phospholipid Metabolism in Macrophages of Phosphatidylinositol Liposomes FASEB.

AVAILABLE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 300535
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62734A	3M162734A875	AJ	167 WWP6
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/1			

11. TITLE (Precede with Security Classification Code)
(U) Diagnosis and Monitoring of Nerve Agent Intoxication by Clinical Chemistry

12. SUBJECT AREAS
0620 Toxicology 0703 Organic Chemistry

13. START DATE: 82 10
14. ESTIMATED COMPLETION DATE: Cont
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	72
85	3.0	61

19. RESPONSIBLE DOD ORGANIZATION
a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
Top, F H JR
d. TELEPHONE NUMBER (include area code)
202-576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
Division of Pathology
b. ADDRESS
Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
Andersen, G L
d. TELEPHONE NUMBER (include area code)
202-576-2183
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
Baze, W B
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
Hayward, I J

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Lab animals; (U) Cats; (U) [illegible]; (U) Intoxication; (U) Nerve Agent; (U) Cerebrospinal Fluid; (U) Enzyme Changes; (U) Specific Proteins; (U) Clinical Monitoring; (U) Diagnosis

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23(U) To develop methods for reliably and quickly establishing a diagnosis of nerve agent intoxication. Determine effective means of monitoring nerve agent casualties and assessing clinical progress. Establish a profile of clinical chemical changes which will serve as a reference for expedient medical diagnosis, treatment, and disposition of nerve agent casualties in the field. Correlate clinical chemical changes with structural changes in experimental animal models of nerve agent intoxication. Investigate relationships between clinical, chemical, and biochemical changes in serum and cerebrospinal fluid in various experimental models of nerve agent intoxication.

24(U) Serum and cerebrospinal fluid of carnivores, rodents, and nonhuman primates will be examined following nerve agent exposure to determine whether there are characteristic changes in the enzyme (acetylcholinesterase, CPK, LDH, SGOT, and others as indicated) content. Additionally these fluids will be tested for presence of central nervous system-specific proteins which may be released following damage to the brain or spinal cord. The central nervous system will be evaluated using standard neuropathological methods. The results of these enzymatic, biochemical and structural studies will be correlated temporally with clinical, survival and dose data to establish correlative profiles of nerve agent-induced damage to the central nervous system.

25(U) 83 10 - 84 09 A protocol has been developed and approved whereby the intoxication of awake cats by slow intravenous administration of Soman will be further studied. Results of the pilot study conducted in 1983 have been analyzed and were reported at the annual Bioscience Review held at the U.S. Army Medical Research Institute of Chemical Defense. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project 3M162734A875 MEDICAL DEFENSE AGAINST CHEMICAL AGENTS

Work Unit: 167 Diagnosis and Monitoring of Nerve Agent Intoxication by Clinical Chemistry

Investigators:
Principal: Gary L. Andersen, MAJ, VC
Associate: Wallace B. Baze, MAJ, VC
Isaac J. Hayward, CPT, VC

Description:

Technical methods for quickly and reliably establishing diagnosis of nerve agent intoxication are developed and investigated. Efforts are aimed at deriving effective means of monitoring nerve agent casualties and assessing clinical progress in these casualties. Experimentally exposed animals are studied in an attempt to establish a profile of clinical chemical changes which might serve as a reference for expedient medical diagnosis, treatment, and disposition of human nerve agent casualties in the field. Structural changes in tissues of experimentally exposed animals are correlated with clinical chemical changes in blood and cerebrospinal fluid. Studies are designed to determine and define acute toxicological parameters of nerve agent poisoning, as well as delayed neuropathological effects in survivors of acute intoxication.

Progress:

The initial study conducted on this project in FY83 demonstrated that intravenous administration of Soman to awake cats was associated with a consistent set of clinical, laboratory and pathologic findings indicating a comparable state of intoxication for all experimental animals. As a result, the intravenously intoxicated Soman cat was determined to be a convenient, reliable, reproducible, experimental mammalian model for systematic investigation of acute and chronic organophosphate nerve agent toxicity. Results of this study were reported at the U.S. Army Medical Research and Development Command's Fourth Annual Chemical Defense Bioscience Review held at Aberdeen Proving Ground, Maryland from 30 May 1984 to 1 June 1984.

During this reporting period, a protocol has been developed and approved for another study involving intravenous administration of Soman to awake cats. This study, scheduled for early FY 1985, will have the following objectives: 1) characterize the chemical and enzymatic changes occurring in blood and cerebrospinal fluid of cats acutely intoxicated with Soman, specifically at 30 minutes, 6, 12, 24 and 48 hours after intoxication; 2) determine the residual chemical and enzymatic changes in blood and cerebrospinal fluid of cats at 4-14 days after acute Soman intoxication; 3) determine whether degenerative changes occur in the peripheral nervous system of cats at 60-90 days after a single, acute exposure to an LD50 of Soman and if any changes detected correlate with an acute or residual chemical or enzymatic change in blood or CSF.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA305980	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. NEW	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62734A	3M162734A875		WWHG 168
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 82/83-6.2/1			

11. TITLE *(Precede with Security Classification Code)*
(U) Development of Diagnostic and Detection Test Systems

12. SUBJECT AREAS
0603 Biology 0601 Biochemistry 0703 Organic Chemistry

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE Cont	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-house

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		84	0.0	00
c. TYPE	d. AMOUNT	85	1.0	73
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Division of Biochemistry Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)* Washington, D.C. 20307 - 5100	b. ADDRESS Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Gentry, M K
d. TELEPHONE NUMBER *(include area code)* (202) 576-3551	d. TELEPHONE NUMBER *(include area code)* (202) 576-3527
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Doctor, B P g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Lab Animals; (U) Hybridoma; (U) Monoclonal Antibody; (U) Photo-ligands; (U) Diagnosis (U) RAM V (U) Mice

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The technical objective of this work unit is to produce monoclonal antibodies using cell fusion techniques. The antigens to be used are defined molecules involved in pathogenesis of diseases and/or are specific for infectious agents (e.g. enzymes, surface antigens, growth factors, and receptors). Using monoclonal antibodies thus produced the immunospecificity will be defined and specific diagnostic or detection test developed. Later on these tests will be converted to kits for use in the field.

Secondly, it has been possible to develop monoclonal antibodies to enzyme which is irreversibly inhibited by organophosphate. Some of these antibodies show much higher specificity to inhibited enzyme than native enzyme. Using the differential specificity of these antibodies a test system will be developed for detection of organophosphate in environment and in biological system. There is military relevance in this research.

24. (U) Monoclonal antibody-producing cell line supernates as well as ascites fluids will be isotyped. The antibodies will be screened against a variety of antigens and modified antigen to determine immunospecificity. Either DOT ELISA or ELISA will be developed for the identification of diseased state. The infectious diseases to be explored at the present include Dengue hemorrhagic fever, Japanese encephalitis, shigellosis and antibiotic associated colitis.

25. (U) None.

AVAILABLE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA 305981	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT
10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY	62734A	3M162734A875		AL	169	WW18
b. CONTRIBUTING						
c. CONTRIBUTING	CARDS					

11. TITLE (Precede with Security Classification Code)
(U) Atropine Pharmacology and Endocrine Physiology

12. SUBJECT AREAS
0615 Pharmacology 0616 Physiology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	0.0	00
85	3.0	61

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER (include area code)
(202)576-3551

20. PERFORMING ORGANIZATION
a. NAME
Division of Medicine
b. ADDRESS
Walter Reed Army Institute of Research
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
SMALLRIDGE, R C
d. TELEPHONE NUMBER (include area code)
(202)576-3014
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
BUTKUS, N E
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
FEIN, H G

21. GENERAL USE
FINA
MILITARY CIVILIAN APPLICATION H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Lab Animals; (U) Sheep; (U) Pharmacokinetics; (U) Atropine; (U) Drug Metabolism; (U) Human Volunteers; (U) Rabbits; (U) Rats

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code) (U) RAM ✓

23(U) To develop an analytical procedure for measuring atropine in tissues and biologic fluids. To define the bioavailability of atropine under normal conditions and in situations which may affect drug metabolism. To describe the pharmacokinetics and the physiologic, behavioral, and hormonal effects of atropine. To assess central versus peripheral anticholinergic effects on parameters measured. There is military relevance in this research.

24(U) Atropine tissue and blood levels will be measured by radioimmunoassay. Pharmacokinetic studies will be performed in animal and human models to examine effects of routine of administration, hypovolemia, and metabolic perturbations on drug metabolism. Serial blood samples will be obtained for measurement of atropine and pituitary hormones. Central vs. peripheral anticholinergic responses will be examined by using two drug formulations (atropine sulfate and atropine methyl nitrate).

25(U) A sensitive radioimmunoassay was developed under an ILIR project. Three protocols have been approved for studying phamacokinetics in animals and humans. Drug analysis support was provided for a USAMRDC civilian contract. This is a new work unit for the 6.2 research program for medical defense against chemical warfare agents.

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EDITION OF MAR 68 IS OBSOLETE

PROJECT 3E162777A878

HEALTH HAZARDS OF MILITARY MATERIEL

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	DA OB 6484 7. REGRADING	84 10 01 8. DISB'N INSTR'N CX	DD-DR&E(AR) 636 9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62777A	3E162777A878	BB	041 WWJD
b. CONTRIBUTING				
c. XXXXXXXXXX	STOG 82/83-6.2/2			

11. TITLE (Precede with Security Classification Code)
(U) Biological Interactions with and Hazards of Microwave Radiation

12. SUBJECT AREAS
0618 Radiobiol 0616 Physiol 0705 Rad Chem

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
71 07	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	3.0	814
85	1.5	872

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
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c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER (include area code)
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
Dept of Microwave Research
b. ADDRESS
Div of Neuropsychiatry
Washington, D.C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
LARSEN L E
d. TELEPHONE NUMBER (include area code)
(202)-576-3615
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
HUNT, E L
g. NAME OF ASSOCIATE INVESTIGATOR (if available)

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Microwave Hazards; (U) Bioeffects; (U) Dosimetry; (U) Biophysics; (U) Military Medicine; (U) Psychology; (U) RAM III

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To provide technical and medical information to the Surgeon General, system developers and agencies responsible for safety standards in order to protect the health and effectiveness of military units and affected civilian populations in microwave and RF environments. This requires analysis of the biophysics and bioeffects attributable to non-ionizing radiation under laboratory conditions which reasonably simulate and/or predict operational exposures.

24. (U) To perform basic and applied research on the problem of microwave and RF interactions with biosystems at all levels of analysis from the cellular and molecular to metazoan physiology, pathophysiology and behavior. This requires development of measurement systems for dosimetric analysis, in vitro and in situ; the evalution of frequency, power level, polarization and modulation as important parameters of the radiation; and the use of low level energy to asses the functional state of cells and tissues.

25. (U) 83 10 - 84 09 The development of the 1300 MHz high peak power transmitter is complete. The previous findings of increased ocular lens pathology in vitro with pulsed exposures as compared to continuous wave exposures of equal average power have been confirmed and extended. Furthermore, a distinctive energy-per-pulse dependance was shown in the damage threshold for pulse-modulated exposure. New theory and experimental results are available which relate air and tissue pressures to microwave pulse parameter. A new program for correction of refractive errors in dosimetric images has extended first order diffraction results to higher orders and includes dissipative media. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83 - 30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

Project 3E162777A878 HEALTH HAZARDS OF MILITARY MATERIEL

Work Unit 041: Biological Interactions With and
Hazards of Microwave Radiation

Investigators.
Principal: COL Lawrence E. Larsen, MD
Associate: Edward L. Hunt, B.A.

Introduction

The major features of the microwave program in the subject fiscal year have been the continuation of building problems at the Forest Glen Microwave Exposure Facility and the failure to recruit engineering staff. The latter has reached crisis proportions inasmuch as no engineering staff remains. In the last four years, three engineers have left the department for higher paying position in industry.

The facility problems at Forest Glen represent a continuation of a MCA begun in 1977 which is still not complete. The major deficiencies with respect to the MCA are failure of environmental control in the four microwave exposure chambers. Additional facility problems fall into the domain of the post Engineer. These consist of architectural, mechanical, and electrical deficiencies which have been the subject of work and discussion since the time of beneficial occupancy in 1979. They remain unresolved in spite of attempts to have the deficiences corrected by the DFEA. A decision was made that the scope of work exceeded the capacity of DFEA resources. This led to the formulation of a plan for contract maintenance. In spite of a germinal period of nearly three years that plan has yet to be implemented. Some progress has been made in that the eight animal modules at Forest Glen are operative. Of the four exposure chambers, two are padlocked for moth-ball storage, one is near completion and one is being used in spite of HVAC control.

Dosimetry

The major areas of progress in this program element consists of the further development of DART (Dosimetric Analysis by Radio-frequency Tomography) data

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acquisition system, and numerical analysis diffraction based methods for recovery of resolution in the direction of propagation. The objective is to provide a map of the spatial variations in microwave energy dissipation and energy storage in specific target organs as functions of frequency and polarization.

The DART data acquisition system now includes a scanner with three linear axes and one rotary axis. The position encoders and servomotor drives as well as their respective digital interfaces have been completed. In addition, the microwave network analyzer has been modified to accommodate a faster processor/controller and the microwave program library has been modified to take advantage of the new processor/controller.

The numerical analysis program has demonstrated the unsuitability first order Born and Rytov approximations in tomograph when the relative dielectric constant varies by more than 10-20 percent. The diffraction based methods selected for further development include higher order Born and Rytov methods as well as generalization to the case of lossy media. The major difficulties remaining are related to the numerical accuracy of the results under the normal assumption that the target is weakly scattering. The limitations of the first Born and first Rytov approximations were investigated in lossy media. It was found that the Born case was better than the Rytov case (contrary to what one might be led to expect from the literature); by a wide margin.

Pulse Power

The objective in this technical area is to investigate the limitations of the present safety standard wherein pulse modulation is ignored. The major activities in this program area were continuation of the studies of ocular lens in vitro and the development of a high peak power exposure system for use with in situ preparations. Portions of the millimeter wave Annual Report are pertinent to this work area, and the reader is referred to that section for further discussion.

The ocular lens studies have expanded and confirmed the earlier findings that pulse modulated microwave power with appropriate selection of pulse parameters does produce histopathology with qualitative and quanitative distinctions from continuous wave (CW) exposure of the

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same average power. Recent work has expanded these findings to biochemical endpoints, but data analysis is not yet complete enough to present any conclusion.

The high peak power transmitter had a design goal of 1,500 kilowatts of RF power output over a pulse with range from 1 to 100 microseconds. The major step to reach that goal has been the design and construction of a hard tube modulator to switch the required 4 million watt video pulses needed to accomplish RF amplification. In addition, we have successfully operated the redesigned modulator (the Mark IV version) to switch five million watt video pulses over the required range of pulse widths and duty factors. RF testing has demonstrated 1,806,000 watts of RF output power with the Mark IV modulator. Other aspects of the system are completed as detailed in previous reports, and we plan to start in vitro studies with a custom designed transmission line exposure system in 1st Quarter, FY 85.

Behavior

This program area is essentially indolent. Much of the reason is the aforementioned facility problems at Forest Glen which have denied the program any free field exposure facility for six years.

PUBLICATIONS

1. Guo, T.C.; Guo, W.W.; Larsen, L.E., "A Local Field Study of a Water Immersed Microwave Antenna Array for Medical Imagery and Therapy", IEEE Transaction Microwave Theory and Technique, MTT-32, p. 835, 1984.

2. Guo, T.C.; Guo, W.W.; Larsen, L.E., "Microwave Induced Thermoacoustic Effect in Dielectrics and its Coupling to External Media - A Thermodynamic Formulation", IEEE Transaction on Microwave Theory and Technique, MTT-32, p. 835, 1984.

3. Stanley, M.; Kak, A.C.; Larsen, L.E., "Limitations of Imagery with First Order Diffraction Tomography", IEEE Transaction Microwave Theory and Technique, MTT-32, p. 860, 1984.

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4. Stewart-DeHaan, P.J.; Creighton, M.O.; Larsen, L.E.; Jacobi, J.H.; Sanwal, M.; Galsworthy, P.R.; Baskerville, J.C.; Trevithick, J.R., "Reciprocity Between Duration of Exposure and Specific Absorption Rate for Cataractogenesis Induced by Pulsed Microwaves", *Invest. Ophthalmol. 25* (supplement No. 3), p. 139, 1984.

5. Creighton, M.O.; Hanington, A; Trevithick, J.R., "*In vitro* Effects of Elevated Temperature on the Cornea", *NATO Advanced Research Workshop and 5th International School of Pure and Applied Biostructure, Erice, Italy*, Abstracts, p. 23, September 1984.

6. Creighton, M.O.; Stewart-DeHaan, P.J.; Sanwal, M.; Trevithick, J.R., "Studies on Elevated Temperature on the Rabbit Cornea *In vitro*", *Cell Biophysics*, in press, 1984.

7. Creighton, M.O.; Trevithick, J.R., "Effects of Elevated Temperature on the Cornea *In vitro*", *Int. Soc.* Eye Res. III, p. 89, 1984.

8. Trevithick, J.R.; Creighton, M.O.; Stewart-DeHaan, P.J.; Sanwal, M., "Reciprocity Between Duration and Power for Pulsed Microwaves in Cataractogenesis", *Oral Presentation at NATO Advanced Research Workshop, Erice, Italy*, September 1984.

9. Stewart-DeHaan, P.J.; Creighton, M.O.; Larsen, L.E.; Jacobi, J.H.; Sanwal, M.; Baskerville, J.C.; Trevithick, J.R., "*In vitro* Studies of Microwave-Induced Cataract: Reciprocity Between Exposure Duration and Dose Rate for Pulsed Microwaves", *Exp. Eye Res.*, in press, 1984.

10. Linklater, H.A.; Galsworthy, P.R.; Stewart-DeHaan, P.J.; Trevithick, J.R., "The Use of Guanidinium Chloride in the Preparation of Stable Cellular Homogenates Containing ATP", *Anal. Biochem.*, submitted for Publication, 1984.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OC 6472
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62777A	3E162777A878	AB	042 WWIA
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/?			

11. TITLE (Precede with Security Classification Code)
(U) Non-Auditory Effects of Blast Overpressure

12. SUBJECT AREAS
[illegible] Weapons Effects 0617 Protective Equipment 0619 Stress Physiology

13. START DATE: 78 03
14. ESTIMATED COMPLETION DATE: Cont
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-house

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	5.0	312
85	5.0	333

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
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Washington, D. C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
Top, F H Jr
d. TELEPHONE NUMBER (include area code)
202/-576-3551

20. PERFORMING ORGANIZATION
a. NAME
Department of Respiratory Research
Division of Medicine
b. ADDRESS
Walter Reed Army Institute of Research
Washington, D. C. 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
Phillips, Y Y
d. TELEPHONE NUMBER (include area code)
(301)427-5380
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
Hoyt, R F
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
Dodd, K T

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Volunteers ;(U) Impulse Noise; (U) Blast Overpressure; (U) Pulmonary Physiology; (U) Gastro Intestinal Physiology; (U) RAM III; (U) She (U) Lab animals

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To define the physiologic effects of blast overpressure (BOP) exposure upon humans. To develop a laboratory model of blast injury. To assist in special studies of weapon specific BOP at the direction of HQ, USAMRDC.

24. (U) Approach includes use of a high velocity water jet of discrete impulse to model blast effects on large animals. Pathologic and lung parenchymal function comparisons of water jet and free-field blast injury will be made. Chronic effects of repeated BOP exposure will be assessed in man and animals. Pathophysiologic events of blast injury will be monitored by implanting transducers.

25. (U) 83 10-84 09 Completed study delineating the properties and flow rate of lung lymph following threshold injury blast. Designed and participated in a field study to characterize the risks of crew injury from blast and oxides of nitrogen inside the M2 Bradley Infantry Fighting Vehicle and M113 Armored Personnel Carrier defeated by high explosive anti-tank (HEAT) rounds. Assisted in evaluation of BOP health hazards through participation in Evaluation Coordination Working Group or Test Integration Working Group for M109 Self Propelled Howitzer Improvement Program, K6 120mm Tampella Mortar, L119 105mm British Light Gun and AT-4 Swedish medium anti-tank weapon. Conducted collaborative experiments with French scientists on blast injury and generalizable damage risk criteria. Prepared the summary of nonauditory research for NATO Panel VIII Research Study Group 6 ("Effects of Impulse Noise"). Using upper respiratory contusion as a marker, determined the threshold for nonauditory injury for large animals (sheep and swine) for simple Friedlander type blast waves. Because of the increasing Army requirement for guidance on blast overpressure exposures above the present limits (MIL-STD-1474B), a volunteer study has been designed: "Direct Determination of Exposure Limits for Intense Freefield Impulse Noise". For technical report see Walter Reed Army Institute of Research Annual Progress Report, 10 Oct 1983 thru 30 Sept 84.

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PROJECT 3E162777A878 HEALTH HAZARDS OF MILITARY MATERIEL

Work Unit 042: Non-Auditory Effects of Blast Overpressure

Principal Investigator: Yancy Y Phillips, M.D., MAJ(P), MC

Associate Investigators: Robert F. Hoyt, Jr., D.V.M., M.S., MAJ, VC
Thomas G. Mundie, Ph.D., CPT, MSC
Kenneth T. Dodd, Ph.D., GS-12

Problem Statement and Objectives

Certain Army weapon systems currently in use and others still in development produce levels of blast overpressure which exceed the limits defined in MIL-STD-1474B "Noise Limits for Army Materiel". The research objective of the Department of Respiratory Research is to define the risk of non-auditory injury to crew members from the blast overpressure produced by these weapon systems. Toward this end, experiments are conducted to obtain various types of physiological and biophysical data which can be related to injury to the larynx, trachea, lungs or gastrointestinal tract. The overall goal of the WRAIR program is the development of generalizable criteria for the assessment of the health hazard of impulse noise and complex wave environments.

Progress and Accomplishments in FY84

1. A major joint study by the Walter Reed Army Institute of Research and the US Army Ballistic Research Laboratory (BRL) was conducted at Aberdeen Proving Ground (APG), MD, utilizing large animals to assess the potential for nonfragment casualties in the M113 armored personnel carrier and M2 Bradley infantry fighting vehicle when penetrated by high explosive anti-tank munitions. The Department of Respiratory Research contributions included the development of the rationale for the study, formulation of the experimental design, coauthoring of the protocol, development of the necessary experimental methodology, and the training and supervision of laboratory technicians in animal handling and restraint techniques. The Department has taken the lead in coordinating data analysis with BRL and in producing a motion picture documenting the study.

2. Original injectable anesthetic regimens were developed for both swine and sheep for use in the blast behind aluminum armor field study held at APG. These new regimens provided up to 1.5 hours of painfree restraint with a single intramuscular injection and will be a useful addition to the scientific literature on animal chemical restraint.

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3. In preparation for the APG study, a member of our research group visited British scientists at the Chemical Defence Establishment (CDE), Porton Down, England, to review the British experiments which precipitated the blast behind aluminum armor controversy. To assure that the results of the U.S. study could be validly interepreted with respect to the earlier CDE work, British scientists observed part of the ongoing APG study.

4. Coordinated with the Combat Systems Test Activity (CSTA), Chemistry Section and the BRL to assure proper toxic gas data collection and interpretation in the behind aluminum armor study. U.S. and U.K. field test toxic gas data do not agree and the Department coordinated with U.K. scientists to allow participation of CSTA and WRAIR personnel in a U.K. field test in December 1984 in order to compare different methodologies in the same toxic gas atmosphere.

5. The Department of Respiratory Research has provided briefings and information papers on BOP for the highest levels of Army leadership. In FY84 we presented MRDC positions on blast biology research to the Commander, DARCOM (now AMC); the Under Secretary of the Army; and the Army Science Board.

6. The mission of the Department has been expanded to include consideration of the combat casualty care aspects of pulmonary injury. A new work unit, Pulmonary Blast Casualty Management, was developed and supporting manpower requirements were detailed for the WRAIR Manpower Survey.

7. Represented MRDC at Evaluation Coordination Working Group (ECWG) meetings for the L119 105mm British Light Gun. The L119 is being procured as the new artillery canon for the Light Divisions. Evaluated data for the Israeli K6 120mm Tampella mortar and prepared a noise hazard assessment of the mortar and recommendations for further testing. Represented the MRDC at the third Test Integration Working Group (TIWG) meeting for the AT-4 shoulder-fired light anti-tank weapon (LAW) and offered guidance on blast overpressure (BOP) data collection in the Detailed Test Plan. Consulted on a BOP test design for the M109 Self-Propelled Howitzer Improvement Program (HIP).

8. Lovelace Medical Foundation has decided to disassociate themselves from blast research now underway in an ongoing MRDC contract. The Los Alamos National Laboratories (LANL) has agreed to manage the government-owned-contractor-operated (GOCO) facilty at Kirtland AFB. The Department has overseen a smooth transfer from Lovelace to Los Alamos with consideration of personnel, equipment, veterinary support, etc.

9. A protocol was designed to elucidate a generalizable damage risk criteria for non-auditory exposure to Friedlander freefield blast waves. The objective of this study is to determine a threshold for laryngeal injury in terms of peak pressure and impulse in sheep and swine exposed to 5, 25 and 100 repetitions of freefield blast. This will serve as an absolute limit on human exposure.

10. Because of the critical Army requirement for definitive guidance on BOP exposure limits for mortars and artillery, we have designed a volunteer study to directly determine exposure limits for intense freefield impulse noise. This will be done on contract at the Los Alamos National Laboratory in New Mexico and will be supervised by WRAIR and USAARL. The study will last at least 2 years and will expose volunteers to increasing level and number of blasts above the Z-curve of MIL-STD-1474B to one of several stopping points.

11. Represented US interests at NATO Panel VIII RSG-6 ("Effects of Impulse Noise") fifth meeting in Meppen, FRG. Prepared summary report on nonauditory injury from intense impulse noise which was accepted by RSG-6. Reviewed MRDC plans to conduct a large scale volunteer study to determine new impulse noise exposure standards.

12. A study of animals for a protocol assessing the influence of blast on pulmonary vascular permeability at threshold pulmonary injury was completed. Preliminary data analysis revealed that there is no blast-induced increase in pulmonary vascular permeability as evidenced by lung water and solute flux at levels of blast where minimal, grossly detectable pulmonary injury is observed.

13. Participated in a review of the USAMBRDL contract program to reevaluate carbon monoxide exposure limits for US Army vehicles.

14. Work was sponsored at Lovelace on a protocol from the WRAIR Division of Pathology entitled: "Acute Gross, Histological and Ultrastructural Effects of Multiple Exposure to Blast on the Rat Respiratory System." The Lovelace group also completed a protocol by USAARL entitled: "Physical Measurements of Noise. Attenuation of Hearing Protective Devices for Typical Impulse Noise."

Recommendations and Objectives for FY85

1. Begin the volunteer study in coordination with USAARL and Los Alamos National Laboratory to directly determine exposure limits for intense freefield impulse noise.

2. Complete analysis of overpressure traces inside M113 and M2 AFVs and design follow-on experiments.

3. Complete analysis of oxides of nitrogen (NO_x) levels inside defeated AFVs and participate in UK studies. Design a program to address toxicity of militarily relevant NO_x exposure scenarios.

4. Continue study of the role of cloth ballistic vest in primary blast casualty production. Using mathematical model and animal studies, investigate concepts for practical blast protection.

5. Evaluate the effects of the new Explosive Ordnance Disposal Suit in a primary blast environment.

6. Develop a pulmonary contusion model for laboratory use in simulating casualty level blast lung injury.

7. Establish critical variables for injury (eg. luminal contents, prestress, bubble size) in an in vitro model of gastrointestinal blast injury.

8. Continue TIWG and ECWG participation for various Army weapon systems.

PUBLICATIONS

1. Phillips, Y.Y, Jaeger, J.J., Laube B.L. Rosenthal, R.R.: Eucapnic voluntary hyperventilation of a compressed gas mixture: a simple system for bronchial challenge by respiratory heat loss, American Review of Respiratory Disease (in press).

2. Young, A.J., Jaeger, J.J., Phillips, Y.Y, Hoyt, R.F., Jr., Yelverton, J.T. Fletcher, E.R. and Richmond, D.R.: Intrathoracic pressure in humans exposed to short duration airblast (submitted for Publication).

3. Hoyt, R.F., Jr. and Withrow, S.J.: Oral malignancy in the dog. Journal of American Animal Hospital Association 20(1): 83-92, 1984.

4. Hoyt, R.F., Jr.: Cricothyroidotomy and Trachestomy Techniques. In Emergency War Surgery Training Manual, 1984.

5. Seim, H.B. and Hoyt, R.F., Jr.: Veterinary Cryosurgery. In Textbook of Small Animal Surgery. Edited by Courley, I.M. and Vasseur, P.B., J.B. Lippincott Co., Philadephia, PA. (in press).

6. Verma, Pritam S., Hoyt, R.F., Jr., Jackson, A.J., and Phillips, Y.Y: Pharmacokinetics of intravenously administered desmosine in sheep. Connective Tissue Research (in press).

7. Young, A.J., Jaeger, J.J., and Phillips. Y. Y "The Influence of Airway pressure on lung injury resulting from airblast", Military Medicine (in press).

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PROJECT 3E162777A879

MEDICAL FACTORS ENHANCING SOLDIER EFFECTIVENESS

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA OC 6453	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT
10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY	62777A	3E162777A879		AA	041	WWJ2
b. CONTRIBUTING						
c. XXXXXXXXX	STOG 82/83-6.2/2					

11. TITLE (Precede with Security Classification Code)
(U) Military Preventive Psychiatry

12. SUBJECT AREAS
0605 Clinical Medicine 0510 Psychology 0616 Physiology

13. START DATE 76 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
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EXPIRATION
b. CONTRACT/GRANT NUMBER
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d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	8.0	838
85	8.0	963

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Neuropsychiatry
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR Marlowe, D H
d. TELEPHONE NUMBER (include area code) (202)576-3551	d. TELEPHONE NUMBER (include area code) (202)427-5312
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) Ingraham, L H g. NAME OF ASSOCIATE INVESTIGATOR (if available) Jones, F D

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23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) This unit examines the dynamics of those specific factors within military organizations and environments that are conducive to psychiatric illness, operate to produce psychiatric casualties and lead to the generation of dysfunctional behaviors and decrements in military performance. These studies have direct relevance for the development of programs of imtervention and prevention and the development of effective techniques for the prevention of psychiatric casualties & behavioral dysfunction. 24. (U) The methods of clinical psychiatry, social and clinical psychology, social anthropology and field epidemiology are used to identify factors that generate psychiatric casualties, behavior dysfunction and performance dysfunction and decrement in order to modify such factors or the relationship between them. 25. (U) 83 10 - 84 09 Studies of the health problems of deployment, and of spouses of soldiers who have deployed have been completed. These have demonstrated the importance of command directed programs for family support for decreasing a variety of medical and behavioral problems in soldiers and their families. A study of delayed stress response in Vietnam veterans was completed, along with a bibliography of behavioral science references based on that war. A handbook of Military Psychiatry is being written. Collaborative studies with the Israeli Defense Force (IDF), to include a cross national measure of morale, are being conducted to gain a cross-cultural perspective on the development of morale and readiness. A study of the life cycles of career NCO's, with emphasis on health and stress risk, is in progress. An extensive study of Infantry Division (Light) soldiers and families is in progress. This is to help determine how military espirit and cohesion are developed at the large unit level, and the relation they have to family adjustment and satisfaction with the military. A study of female soldier bonding and stress is being developed. Studies of the stress of combat and soldier and medical effectiveness in Operation Urgent Fury are being analyzed. A study of family adaptation to units in USAREUR continues. For further details, see WRAIR Annual Progress Report, 1 Oct 83 - 30 Sep 84.

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Project 3E162777A879 MEDICAL FACTORS ENHANCING SOLDIERS EFFECTIVENESS

Work Unit 041 Military Preventive Psychiatry

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Segal, Ph.D.; Mady Segal, Ph.D.; Reuvan Gal, Ph.D.;
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S. Dank; SP5 Veronica L. Davis; SP5 Dawn D. Caban; SP5
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Description

Neuropsychiatric casualties have represented a major source of manpower loss in every armed conflict in which the United States Army has been involved. Further, in times of peace the Army suffers significant personnel losses and costs as a function of behavioral dysfunctions, performance decrements, effectiveness deficits, psychosomatic illnesses, psychogenically based disorders and neuropsychiatric diseases. Many of these losses and costs appear to involve predisposing risk factors that are parts of the general and human ecology of the Army. Unique aspects and demands of military life engender both strains and stresses that further the risk of the individual and the group for dysfunctional and ineffective behavior. The symptomatic and often costly responses to stressful aspects and demands in the military are in part determined by the health status and coping styles of the individual and in part by the social milieu in which stressful events are experienced. This unit examines the interaction of the individual and group within the special ecological settings of the Army, ranging from the intense, life-threatening multiple stresses of combat to the daily stresses and strains of garrison and training, examines the dynamics of those specific factors within the military organizations and environments that contribute to psychiatric illness and psychiatric casualties, and which lead to ineffectiveness, the generation of dysfunctional behaviors, and decrements in military performance.

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DELAYED STRESS RESPONSE SYNDROME AND PATTERNS OF PSYCHOSOCIAL SUPPORT

The purpose of this study was to determine the incidence of post traumatic stress disorder (PTSD) among Vietnam veterans, and factors which influence its prevelence. Results of the first three phases of the project indicate that the incidence of PTSD among veterans who have maintained a military affiliation is significantly lower than that reported for veterans in the civilian sector.

Results of analyses of factors influencing the development of PTSD revealed that combat experience and social support both during and after service in Vietnam were the best predictors of PTSD. Data analyses are complete and a final report is being written.

PATTERNS OF PSYCHIATRIC NEED AND INTERVENTION AMONG US ARMY TROOPS DURING THE VIETNAM CONFLICT

The purpose of this study was to determine the patterns and role of various treatments for psychiatric problems in Vietnam. Pychiatrists' responses regarding treatment and recoverability of soldiers experiencing combat stress confirmed the validity of the conventional principles of "immediacy", "expectancy", and "proximity". Especially notable was the impression that many division level psychiatrists knowingly returned soldiers to combat duty under the influence of psychoactive medication despite being uncertain as to whether or not the medication in question would enhance or degrade combat performance.

Secondary and tertiary prevention activities consumed most of the psychiatrists' time and energy. Although the majority identified psychosocial and environmental factors as predominant in the etiology of combat breakdown, only those psychiatrists with either training in the military or prior military experience seemed eager and confident in providing primary prevention care (i.e. command consultation).

GRENADA LESSONS LEARNED

The impact of combat stress, cohesion, psychological readiness, medical readiness, and joint operations on combat effectiveness were examined by means of interviews with combat soldiers who participated in Operatin Urgent Fury in Grenada. Interviews with soldiers of the Ranger battalions and the 82nd Airborne Division have been completed. A paper on medical considerations in the operation is currently in draft, and analyses of other topics continue.

SINAI PEACEKEEPERS

This research was conducted to examine military attitudes

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concerning "peacekeeping", since this role seems likely to be required of the US Army in the future. Such attitudes are important as negative views (as some suggested would be found) of the job could help increase stress and decrease commitment leading to morale and attrition problems and possibly abuse of the peacekeeping role. The study found that paratroopers from an elite combat unit generally adapted well to the role of peacekeepers, although several recommendations were made to improve their level of coping and to decrease family adjustment problems. A final report has been written.

MILITARY PSYCHIATRY HANDBOOK

This handbook is aimed at presenting current theory and practice of military psychiatry in combat operations to military mental health and other medical professionals. Sections will include chapters on the principles of diagnosis and treatment; utilization of mental health resources, and psychophysiology of combat stress; and considerations in future combat including such special issues as combat sequelae and training personnel.Approximately three-fourths of the book is in final draft.

AMINO ACIDS/SLEEP ALERTNESS STUDIES

Since high intensity, mobile future combat requiring dispersion of forces may invalidate the psychiatric principles of treatment proximate to the battle front and return of the soldier to his own unit, and since continuous combat may markedly disrupt scheduled rest, the development of drugs or nutrients to combat anxiety in the unit and to regulate sleep/awake cycles may be important in both the prophylaxis and treatment of combat breakdown. Certain amino acids have potential sedating (L-tryptophan) and alerting (L-phenylalanine, L-tyrosine) properties without the usual risks of sedating and stimulating drugs. Protocols are being developed to test the feasibility of using these amino acids to regulate the sleep/awake cycle.

VIETNAM BEHAVIORAL SCIENCE ANNOTATED BIBLIOGRAPHY

The purpose of this is to provide a review and summary of all relevant behavioral and social science publications relating to ground troops of the war in Vietnam. Over 300 citations have been collected and it is expected that a final report will be produced by end of FY 85.

SENIOR NCO STRESSORS

The study examines stress factors among senior non-commissioned officers. The objective is to understand the stresses and strains in the NCO role which contribute to individual and organizational dysfunction. The method involves the gathering of narrated career histories which is a modification of life history techniques used in social anthropology, sociology, and oral history.

A number of themes have emerged for analysis, including leadership modes, how jobs and job responsibilities are viewed by different ranks, ethical standards and NCO - officer relationships. Interviews and transcribing are in progress.

IDL FAMILY LIFE EVALUATION/IDL MILITARY UNIT EVALUATION

At the request of the DCSPER, Army (New Manning System Task Force), a multi-year study of the human dimensions of the 7th Infantry Division-Light (IDL) will be conducted. The study's purpose is to evaluate the impact of changes in organizational structure, weapons and weapons systems, and doctrine upon perceived combat readiness, general well-being, morale, and cohesion among soldiers and their families. The results of the study will be used to establish policies in the creation of future "light" infantry divisions. The study is in the protocol and instrument development stage, with pretest of instruments scheduled for FY 85.

CROSS-NATIONAL MEASUREMENTS AND CORRELATES OF ARMY UNIT MORALE

An Israeli morale questionnaire was translated into English and pre-tested on a US Army sample. The final version was then given to two American airborne battalions and two cavalry squadrons (one in Germany and one in CONUS). Indices of unit effectiveness were also collected to include judgements of key leaders, disciplinary actions, reenlistments, physical training test scores, SQT results, and sick call rates.

Considerable similarity was found between Israeli and US units in terms of the main factors underlying morale. Further, US units proximate to troops classified as possible future enemies (i.e., the border of Germany) were more similar to Israeli units than to US units in the CONUS. Final reports are being written.

CORPORATE FITNESS/STRESS MANAGEMENT IN THE ODCSPER

Data gathering to evaluate the ODCSPER Corporate Fitness program and stress management interventions was completed during FY 84. This evaluation was requested by The DCSPER, U.S. Army. The final data base for the evaluation consists of five waves of questionnaires and psychological instruments articulated to the major events of the Corporate Fitness program. The instruments measured a number of perceptions and attitudes ranging from "well being" through perceived health status, job and workplace stress, perceptions of change in self and in the workplace.

Significant relationships were found between perceived levels of stress, symptom prevalence, job satisfaction, relationships to supervisors and social supports available to the respondent.

Analysis of the data continues and a final report will be completed in FY 85.

FT. HOOD MILITARY COMMUNITY STUDY

A Cavalry Division requested a community study to develop an appreciation of the life stresses having an impact on the spouses of servicemembers.

This was a survey study based on a large stratified sample of community residents (spouses). It was found that military life stress is related to social support, which has a direct and moderating effect on the stress-outcome relationship.

COMBAT THEATRE STRESS STUDIES

Since the United States is not currently engaged in armed conflict, the best data on adaptation to combat comes from past combat experience and current combat by other nations. Past combat experience is being studied through the development of cmprehensive bibliographic material from various wars and review of medical experience recorded in medical records available from Vietnam.

The current or recent combat experience of foreign nations, particularly Israel, is being studied through personal contacts and joint projects. Contacts made through the Military Section of the World Psychiatric Association have resulted in information on the experience of Iranian psychiatrists in the Iraq-Iran War and of British and Argentinian Psychiatrists in the Falklands War. The lessons of these wars and of the US experience in the Grenada Rescue Mission are being prepared for dissemination.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

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9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62777A	3E162777AB79	AA	042 WWJ3
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 82783-6.272			

11. TITLE (Precede with Security Classification Code): (U) Military Psychiatric Epidemiology
12. SUBJECT AREAS: 0605 Clinical Medicine 0510 Psychology
13. START DATE: 76 07
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House
17. CONTRACT/GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	5.0	522
85	5.0	709

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code): Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL: Top, F H Jr
d. TELEPHONE NUMBER (include area code): (202) 576-3551

20. PERFORMING ORGANIZATION
a. NAME: Walter Reed Army Institute of Research Division of Neuropsychiatry
b. ADDRESS: Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR: Marlowe, D H
d. TELEPHONE NUMBER (include area code): (202) 427-5312
f. NAME OF ASSOCIATE INVESTIGATOR (if available): Camp, N M
g. NAME OF ASSOCIATE INVESTIGATOR (if available): Schneider, R J

21. GENERAL USE: FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Psychiatric Illness; (U) Military Adjustment; (U) Environmental Factors; (U) Social and Psychological Factors; (U) Stress; (U) RAM III

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) This unit examines military organizational, social, psychological, and environmental factors that create risk for and are conducive to psychiatric disease, psychosomatic illness, behavioral dysfunction and physical illness as they affect Army personnel and have an impact on care giving agencies. 24. (U) The methods of epidemiology, including records surveilllance, population and demographic analysis, questionnaire and field and cohort studies as well as methods of the psychological and social sciences are used to delineate environments of risk for psychiatric illness and periods of special risk for such illness at critical points in the career of the soldier. 25. (U) 83 10 - 84 09 A study of the epidimiology of health problems of female soldiers has been completed. This is one part of a series of studies designed to a) determine special risk factors for health & behavioral problems, and b) suggest interactions based on changing organizational training, doctrine and mission priorities. Other ongoing studies in this series are the epidimiology of suicide in the US Army, minority group stress, high frequency users of health care facilities, and stress in Special Forces troops and their families. An extended study of selected COHORT units is underway. This is to determine if a fundamental restructurng of how military units and communities are formed can be used do increase soldier commitment, readiness, and willingness to fight. An evaluation of a Corporate Fitness program carried out at the Army General Staff and at a Divisional Staff has been completed. A normative base for the General well being scale in the Army was completed. Studies of well being of female personnel in selected military units were completed. Analysis of data examining drill sergeant stress and mediating and risk factors continues. Data analysis of modes of psychiatric treatment in Viet Nam continues. Studies of the basis and diagnosis of combat stress breakdown, in collaboration with the Israeli Defense Force, is in final stages of development. For further details, see WRAIR Annual Progress Report 1 Oct 83 - 30 Sep 84.

Project 3E162777A879 MEDICAL FACTORS ENHANCING SOLDIERS EFFECTIVENESS

Work Unit 042 Military Psychiatric Epidemiology
Investigators.
Principal: David H. Marlowe, Ph.D.
Associate: COL Franklin D. Jones, MC; COL Norman M. Camp, MC; COL Jesse J. Harris, MS; LTC Edwin W. Van Vranken, MS; LTC Larry H. Ingraham, MS; MAJ Stanley Holgate, MS; MAJ James A. Martin, MS; MAJ Terrence D. Fullerton, MS; MAJ Theadore P. Furukawa, MS; MAJ Robert J. Schneider, MS; CPT James E. Griffith, MS; CPT Robert H. Stretch, MS; CPT Kathryn H. Knudson, MS; Joseph M. Rothberg, Ph.D.; Charlene S. Lewis, Ph.D.; Mary L. Lozano, Ph.D.; David Segal, Ph.D.; Mady Segal, Ph.D.; Reuven Gal, Ph.D.; Caren M. Carney, M.A.; Richard Howard, M.A.; Richard Oldakowski;SFC Edgar N. Marshall; SSG Emily M. Kukura; SP5 Diana L. Smith; SP5 Bonnie S. Dank; SP5 Alvin B. Taylor; SP5 Veronica L. Davis; SP5 Dawn D. Caban; SP5 Gary Killiebrew; SP5 William C. Marshall; SP5 Patricia A. Heagy

Description

The military environment places demands and strains upon its population that are markedly different from those of civilian environments. The demands and differences in terms of individual and unit effectiveness and performance, mental and physical health, and behavioral disruption and dysfunction have chronic effects in peacetime. In periods of deployment and combat, such stresses may have acute effects on the capability of units and individuals to perform their missions. This unit examines military organizational, social psychological, and environmental factors that create risk for and mitigate against psychiatric disease, psychosomatic and physical illness, behavioral dysfunction and disruption of performance as they affect Army personnel and have an impact on health care agencies. The methods of epidemiology, including records surveillance, population and demographic cohort studies and methods of the psychological and social sciences are used to delineate factors conducing to risk as well as mitigation for such illnesses, disruptions and dysfunctions.

SUICIDE IN THE US ARMY

The goal of this study is to determine if there are meaningful fluctuations in the patterning of military suicide data and to identify the correlates of any of these changes.

AVAILABLE

The Army data under analysis are the 834 suicides recorded during calendar years 1975 through 1982. Because the number of suicides in the Army is relatively small (about 105 per year) meaningful conclusions are difficult to draw. Although there are some deviations from the temporal distribution of suicides in civilian populations, military suicides generally appear to involve the same general causal factors, such as loss of a love object. The final report of this study is in preparation and will be available in FY 85.

GENERAL WELL BEING SCALE: NORMATIVE CONSIDERATIONS

The General Well Being Scale has been extensively used in the work of this department; while it has been normed against the overall U.S. population there have been no specific norms available for use in the study of military populations. In order to develop adequate norms for the Army, all known Army samples of the GWB have been merged into a single data base. Psychometric scale analyses and between group comparisons are complete, as is a review of the GWB literature. A series of baseline norms representing the overall combat arms population and selected special populations is now available for comparative analyses. This study is completed and a final report is available.

EPIDEMIOLOGIC FACTORS OF HEALTH OF WOMEN IN THE ARMY

This epidemiological overview of the health and effectiveness of women in the Army has been driven by two observations: -- the female composition of the predominately male US Army is increasing, and the hospitalization rate for females has historically been higher than that of males. Other data suggest that females may not be as well integrated into the normal flow of Army life as males are. For example, 3 year attrition amounted to 38.4% for males and 49.2% for females and the re-enlistment rate for females was 16% compared to 21% for males. Ancillary data collected in the course of various studies suggest that these data can be interpreted as functions of higher levels of stress and distress observable among women soldiers and the lower level of social supports available to women,particularly in heavily male units. Status: Data analyses are continuing.

HIGH FREQUENCY USERS OF ARMY MEDICAL CARE

The purpose of this study is to identify and compare two populations of soldiers - those who apparently overuse military medical care facilities and those who apparently underuse such resources - through the use of both questionnaires and semi-structured interviews. This project has been delayed because of the impending implementation of several pilot electronic outpatient data collection systems by Health Services Command. It is anticipated that this project will be implemented at one or more MEDDACS when these systems are operational.

HEALTH PROBLEMS OF DEPLOYMENT

A. FAMILIES Brief family separations resulting from military training exercises are a common phenomenon in Army communities. Extended separations are less common and therefore little is known about the effects of these separations on Army family members. This study has looked at the effects of deployment separations on military families and active duty members utilizing questionnaires, psychological instruments and interviews. The subjects have been soldiers and family members of the 82nd ABN division.

There is little question that deployment separation creates various degrees of psychosocial disruption and stress for military families. There is also little reason to believe that we will ever be able to totally remove all stresses associated with deployment separation. However, this study provides a number of recommendations to maximize support and minimize such stress and family problems. Most important among these are the establishment of the unit as a base for communication and resource distribution. A final report is available.

B. SOLDIER HEALTH CARE UTILIZATION

The purpose of this study is to present a detailed epidemiological view of the health of the first United States Army battalion deployed as peacekeepers to the Sinai in 1982 as part of the first wave U.S. contribution to the MFO. The focus of this study is on stress as a cause of medical care utilization in the Army. The longitudinal data include measures derived from records of the health care system which were used to examine the health effects of stress associated with several types of transition states.

Among the study findings were elevated rates of sick call in the days prior to going to field outposts. The increased visit rate at the transition to the field paralleled the increased use of health care at the time of deployment, providing evidence that the stress of deployment leads to increased medical visits. A final report is in progress.

FAMILY ABUSE DATA BASE

This is an exploratory investigation of spouse and child abuse. The goal is to provide a profile of the abused family to assist Army social work services in determining prevention treatment needs, as well as to provide basic research data concerning any military specific stresses which may serve to precipitate abuse; preliminary findings suggest that such predisposing stresses might indeed exist. A protocol is being developed to investigate this area in detail.

SPECIAL FORCES STRESS ISSUES

A. SOLDIERS Elite military units have been widely recognized as exceptional fighters and as highly resistent to the stress of battle. This research was designed to determine the tangible benefits of belonging to an elite Army unit in peacetime. We found that such soldiers report better health and more satisfaction than soldiers from non-elite units. These differences seem to be due to higher cohesion and group support. Data analyses are complete and the study is currently in final write-up stage.

B. FAMILY STRESS

The purpose of this study was to examine the possible effect that increased unit cohesion or organizational committment could have on a soldier's family. A 16-module questionnaire was administered to Special Forces soldier's wives. The study is in the data analysis phase. Results will be compared and/or combined with other spouse data being collected by members of the Department of Military Psychiatry. An analysis of the questionnaire will examine the impact made by the husbands' commitment and bonding to the unit in relation to the wives' general well-being. Various other findings are currently under investigation.

FT. BRAGG MILITARY COMMUNITY STUDY

This is one of several research projects designed to learn how the relations between military communities and military families affect health and adjustment of the military family and the service member. This study examined the general issue of quality of life, including specific questions dealing with how families cope and the etiology of adjustment problems. One major finding is a relationship between community structure (rank, density and location of housing) and the occurence of interpersonal problems, a finding which suggests that such communities could be reorganized for better citizen adjustment. A final report has been written.

COHORT STUDIES

A. UNIT COHESION/SOLDIER STRESS As part of the overall evaluation of COHORT units, a special evaluation of COHORT and conventional units in USAREUR is underway. This evaluation looks at the structure of COHORT units and conventional line unit controls and patterns of adaptation and stress among soldiers and their families. During the past year extensive interview studies have been carried out with all COHORT units assigned to USAREUR and with a selected group of control units. In addition questionnaires assessing levels of unit cohesion and morale and soldier assessment of the unit and its climate, as well assessments of soldier stress have been given to the COHORT units and 9 control units. COHORT units and COHORT soldiers on the average are better bonded and more cohesive than their conventional counterparts. Their members tend to perceive themselves and the unit as more trained and skilled and more ready for combat than do the controls. Stress levels among units appear to be directly related to command climate and the perception of and activities of unit leaders. Family adaptation appears to reflect unit adaptation and soldier satisfaction with the unit. These studies continue.

B. FAMILY STUDY This is a HQDA sponsored longitudinal study of the impact of the Army's New Manning System and specifically the COHORT concept on the life adjustment of military families. Using both self administered questionnaires and indepth personal interviews, data are now being collected from a representative sample of COHORT families (17 company sized units representing all the combat arms at a broad range of CONUS and OCONUS locations).

Initial analyses suggest that important supportive relationships have developed among first term wives but that adequate unit and installation welcome and enculturation efforts are severely lacking. The importance of family preparation for small unit (company and battalion) based deployment and OCONUS rotation has been demonstrated. These efforts not only moderate the stress associated with these events, they have also provided a basis for the development of critically important interpersonal relationships among unit wives.

Further it is evident that the attitude of small unit leaders (from battalion commander to platoon sergeant) toward family life issues can have a major impact on the military wife's attitude and adjustment to the duty demands made on her husband and her attitude toward her community and the Army. This study is in progress.

GRENADA WIVES AND FAMILIES

This study was conducted to determine the effects of combat on waiting families. These data were collected by personal interviews with wives whose husbands had either just returned from or were still in Grenada.

Two major findings were: 1) unit-based family networks for mutual support during combat deployments were very effective and 2) the kinds of information which families need to sustain them through combat deployments. A final report has been written.

SINAI WAITING WIVES STUDY

This was part of a series of studies designed to assess the support needs and support available for wives of soldiers deployed overseas. This study focused on the wives' support networks.

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It was found that a productive and effective interrelationship among the formal Army agencies and Army families can be sustained throughout a lengthy (six month) deployment. Results also indicate that the technique is cost-effective from a readiness stand-point during a deployment with no such family support, 15 soldiers were re-deployed (one at a time) because of family problems. During a deployment with a supportive family environment, only one soldier was re-deployed for pre-existing family problems. A final report has been written.

MINORITY GROUP STRESS

Protocols have been written for the related studies of stress and health care utilization among Hispanic and minority soldiers. Through the application of anthropological techniques and survey instruments, we are addressing (1) past and present health problems among Hispanic soldiers, (2) types of stress and stress responses experienced by Hispanic soldiers and their families, (3) the cultural differences that exist within the Hispanic military community, and (4) possible cultural impact on health care provision.

DRILL SERGEANT STUDY

The research project exploring drill sergeant and NCO stress was a comparative study undertaken in response to a request by The DCSPER. The Department was tasked to explore how stress affects attrition rates during basic training.

Analyses of psychological distress and well-being have revealed no appreciable differences between drill sergeants, a group perceived to be highly stressed, and non-drill NCO's at the same training posts. The absence of a study group of NCO's from non-training environments postpones conclusions about the interaction of social and psychological stressors. However, compared to a large civilian sample, NCO's in this study were moderately distressed a situation that is of concern given the long term negative consequences of chronic moderate stress.

A more encouraging finding suggests that social support had pervasive direct ameliorating effects on psychological distress. The group of NCO's with low support however, fell into the severely distressed range. Finally, these findings point to the potential for using social support as a tool for both prevention of and intervention for socio-environmentally induced psychological distress. A final report has been completed.

IDF COLLABORATIVE STUDIES

Two major collaborations with the Israeli Defense Forces are under development, work which also involves collaboration with investigators from the Department of Behavioral Biology, Division

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of NP, WRAIR. In the first of these studies a complete review of the medical records of all combat psychiatric casualties incurred by the IDF in operation "Peace for Galilee" (Lebanon in 1982) will be conducted. A pilot review of a sample of records was carried out in FY 84 and a full scale review of almost 1000 cases of both initial combat reactions and delayed combat reactions and post traumatic stress disorder is scheduled. The major object of this research is to define, if possible, causal patterns and those factors governing the length of time required for adequate treatment and restoration to combat status. In addition, this research is also directed to the adequacy of field reporting forms and techniques in terms of the generation of information necessary to understand the etiology of combat stress reactions, the appropriate response of treatment resources and epidemiological needs of the Army.

The second study under development is a comparative one of Battalion Surgeons and other "gate keepers" in both the U.S. Army and the IDF. This study is designed to examine the knowledge and assumptions about stress, stress related illnesses and combat stress of such individuals and effects it might have on their diagnostic proclivities in different types of combat situations. The role of such assumptions in the decision making process that leads to either return to duty or rear-ward evacuation will be a major research objective.

COMMAND CONSULTATION STUDY

The command consultation studies provide research consultation and guidance to WRAMC resident psychiatrists as well as collaborative research opportunities for the department. The studies' purposes include: to explore line officers' perceptions of the present Army Military Mental Health System, to apply principles of command consultation in military settings and to assess their current effectiveness, and to provide pilot studies from which more rigorous concepts and measurement instruments can be developed and tested in garrison settings.

The data indicate that officers have a basic appreciation for the training and skills of mental health professionals. However, their encounters with such professionals are very infrequent. This latter finding is one of the bases for a reinterest in command consultation. To reach its potential, the military mental health system may require an analysis of where civilian psychiatry models are appropriate or inappropriate, and a commitment to engaging in outreach programs while adjusting to ever-shrinking resources. This study is in progress.

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FEMALE BONDING AND STRESS

The DCSPER, U.S. Army has asked the Department to explore the structure of "cohesion" in all-female military groups and in mixed military groups through use of statistical and ethnographic means at multiple points in the military career of the female soldier and in modal settings that are typical of assignments for female soldiers.

A formal research proposal is in the final stages of formulation.

PUBLICATIONS

1. Carney, C.M. Well Being and Distress Among Drill Sergeants: Civilian and military comparisons and the role of social support. In G.E. Lee T.E. Ulrich (Eds.), Proceedings, psychology in the Department of Defense, ninth annual symposium (pp. 148-152). Colorado Springs, CO: United States Air Force Academy, 1984.

2. Carney, C.M., Fullerton, T.D., Lewis, C.S., Martin, J.A., Oldakowski, R.J. and Van Vranken, E. The well-being of Army soldiers and their families: Methodological, substantive, and technical considerations. In G. E. Lee T. E. Ulrich (Eds.), Proceedings, psychology in the Department of Defense, ninth annual symposium (pp. 138-142) Colorado Springs, CO: United States Air Force Academy, 1984.

3. Furukawa, T.P. Army Leader's Guide on Bicultural Families. ODCSPER, Army. 1984

4. Furukawa, T.P. "Slapping down the myths about combat stress: A Summary of the Key Myths, Facts, and Implications." Submitted to Proceedings, Fourth User's Workshop on Combat Stress, HQ HSC.

5. Lewis, C.S. Supportive structure for waiting wives. Proceedings, Stress and the Military family. Topeka, KA, The Menninger Fondation, 1984.

6. Martin, J.A., The New Manning System and Family Stress. Army Community Service Annual Training Workshop, Arlington, VA, 13 August 1984.

7. Martin, J.A., Life Satisfaction for Military Wives, Military Medicine, (in press 1984).

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8. Martin, J.A. and Carney, C.M. (in press) Life adjustment among military wives in the Army's New Manning System: A description of first wave data. *Proceedings, Stress and the military family*. Topeka, KA: The Menninger Foundation.

9. Rees, R. and Segal, M.W.. "Role Differentiation in Groups: The Relationship between Instrumental and Expressive Leadership." *Small Group Behavior*, Vol. 15, No. 1 (Feb. 1984): 109-123.

10. Rees, R. and Segal, M.W.. "Intra-group Competition, Equity, and Interpersonal Attraction." *Social Psychology Quarterly* , in press.

11. Rothberg, J.M., Cycles of Suicide, in *Proceedings of the Twenty-ninth Conference on Design of Experiments*, U.S. Army Research Office, Research Triangle Park, NC, 1984

12. Schneider, R. J., Wehrpsychologische Untersuchungen. Bundesministerium der Verteidigung, Bonn: W. Germany, 1984

13. Segal, D. R., Harris, J. J., Rothberg, J. M., Marlowe, D. H., Paratroopers as Peacekeepers, *Armed Forces Society* 10(4): 487-506, 1984

14. Segal, M.W., "Women's Roles in the U.S. Armed Forces: An Evaluation of Evidence and Arguments for Policy Decisions." In Robert K. Fullinwider, ed., *Conscripts and Volunteers: Military Requirements, Social Values, and the All-Volunteer Force*. (Totowa, N.J.: Rowman and Allanheld, 1983), pp. 200-213.

15. Segal, M.W., "Enlisted Family Life in the U.S. Army: A Portrait of a Community." In David R. Segal and H. Wallace Sinaiko, eds., *Enlisted Men and Women*. *Pergamon*, in press.

16. Snodgress LL and Schneider, R. J., Assessment of emotional response in cross-cultural research. American Journal of Clinical Biofeedback, 1984 .

17. Stretch, R., and Figley, C. Combat and the Vietnam veteran: Assessment of psychosocial adjustment. *Armed Forces Society*, 10, 311-319. 1984

PRESENTATIONS

1. Carney, C. M., Perceived symptom development among Army nurses assigned to combat or non-combat settings. Paper presented at meeting of the American Psychological Association, August 28, 1984, Toronto, Canada.

AVAILABLE

2. Carney, C. M., The well being of Army soldiers and their families: Methodological, substantive, and technical considerations. Paper presented at Ninth annual symposium of psychology in the Department of Defense, April 18, 1984, Colorado Springs, CO.

3. Carney, C. M., Well being and distress among drill sergeants: Civilian and military comparisons and the role of social support. Paper presented at Ninth annual symposium of psychology in the Department of Defense, April 18, 1984, Colorado Springs, CO.

4. Lewis, C.S. Anthropological interpretations of the General Well Being scale. Paper presented at Ninth annual Symposium of psychology in the Department of Defense. April 18, 1984, Colorado Springs, CO.

5. Lozano, M. L, Methods of studying stress among hispanic soldiers, Paper presented at Naval Post Graduate School, Manpower Research Center, Monterey, California, 1 May 1984.

6 Rothberg, J. M., Cycles of Suicide, Paper presented at 29th Conference on the Design of Experiments in Army Research, Development and Testing, Bethesda, MD 18 - 21 Oct 1983.

7. Segal, M.W., "The Sociology of Military Family Life", paper presented at the Flag Officers Conference, U.S. Coast Guard, Fredericksburg, Virginia, October 5, 1983.

8. Segal, M.W., "Women in the U.S. Armed Forces: Progress and Barriers in the 1980s." Paper presented at the International meeting of the Inter-University Seminar on Armed Forces and Society (IUS), Chicago, October 1983.

9. Segal, M.W., Organizer and Chair. Session on "Military Sociology." Paper presented at meeting of the American Sociological Association, San Antonio, Texas, August 1984.

10. Segal, D. R., Harris, J., Rothberg, J. M. and Marlowe, D. H., Paratroopers as Peacekeepers: Attitude Change in the Sinai. Paper presented at a Inter-University Seminar on Armed Forces and Society, Chicago, IL 21 - 23 Oct 1983.

11. Stretch, R., Post-Traumatic Stress Disorder (PTSD) Among US Army Nurses , Paper presented at annual meetings of the American Psychological Association, August 1984, Toronto, Canada.

AVAILABLE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OC 6457	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
83 10 01	D. Change	U	U		CX	

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62777A	3E162777A879	AB	043 WWJ6
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 82/83-6.2/2			

11. TITLE (Precede with Security Classification Code) (U) Effects of Sustained Operations and Long Range Rapid Deployment upon Soldier Stress-resistance and Performance

12. SUBJECT AREAS
0510 Psychology, 0616 Physiology, 0619 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
78 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
b. CONTRACT/GRANT NUMBER		84	5.0	782
c. TYPE	d. AMOUNT			
e. KIND OF AWARD	f. CUM/TOTAL	85	4.0	265

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Neuropsychiatry
b. ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR KRUEGER, G P
d. TELEPHONE NUMBER (include area code) 202 - 576-3551	d. TELEPHONE NUMBER (include area code) 301 - 427-5521
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) BELENKY, G L g. NAME OF ASSOCIATE INVESTIGATOR (if available) SMITH, H R

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Sustained Military Performance; (U) Sustained Operations; (U) Sleep Discipline; (U) Soldier Stress; (U) Volunteers ; (U) RAM III

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Conduct applied field research oriented to development of recommendations for new doctrine and procedural changes on soldier and commander sleep discipline, work-rest schedules, and countermeasures to operational stressors, to enhance performance, and to prevent or alleviate psychological stress and psychiatric casualties in training, during deployment, and in battle.

24. (U) In collaborative efforts with Army operational and conceptual test units (e.g. OTEA, TRADOC Boards, TCATA, CDEC, ADEA, HEL etc.) design, organize and execute field studies (e.g. OT, FDTE, REFORGER, LID and RDF training missions) on the effects of sustained operations under simulated battle conditions on soldier stress, mood and cognitive performance.

25. (U) 83 10 - 84 09; previously reported under title: Military Stress-Circadian and Ultradian Factors. Progress during FY 84 included implementing modifications to the complex demodulation technique of assessing periodic components in sequentially sampled data to permit assignment of all variance to appropriate spectral components; and development of a speed/accuracy product measure, labeled "Throughput." When data from nine cognitive performance assessment tests were analyzed using this measure, performance degradation functions were statistically identical, attesting to the potential general utility of this measure for analysis of psychological performance data from sustained operations field tests. In collaborative test with Armor Engineer Board, measured psychological stress, mood and performance of tank crews wearing NBC protective clothing in sustained tank operations lasting 24+ hours in 3 test phases. Convened international meeting on sustained military operations research. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project: 3E162777A879 Medical Factors Enhancing Soldier Effectiveness

Work Unit: 043: Effects of Sustained Operations and Long Range Rapid Deployment upon Soldier Stress-resistance and Performance

Investigators:

Principal: Sander G. Genser, M.D. COL MC

Associates: Daniel Redmond, M.D. LTC MC

David Thorne, Ph.D.

Robert Pleban, Ph.D.

Helen C. Sing, M.S.

Problems and Objectives:

This work unit studies circadian and ultradian rhythms in sustained operations and long range deployments, in order to identify the factors that sustain, degrade, or enhance performance in such operations and deployments.

Progress:

Data from subjects undergoing 48-72 hrs of sleep deprivation were analyzed from the viewpoint of dynamic changes in the amount of time spent on performance tasks (PAB, lexical decision task, and grouped shorter tasks) whose requirements were kept constant over the full experimental period. Overall, duty cycle was found to undergo a 25% increase with (1) a disproportionate part of the increase occurring in the latter part of the run and (2) the initially longer tasks being more adversely affected than the shorter ones. Taken together these observations suggest that, with increasing sleep deprivation, the increasing time demand on the subjects required for task completion impinges more and more on their "free" time (time outside of task) and consequently increases the experimental burden. The increasing duty cycle thus becomes part of a self-amplifying feedback loop which magnifies fatigue induced performance decrements. Although the longer tasks appear to contribute disproportionately to this effect, differences among tasks of similar length suggest that length as well as other task variables may be modifiable in such a way as to maximize ability to maintain effective work capacity during continuous operations.

During the 72 hour sleep deprivation study, twenty four hour urine collections were made, and urinary cortisol, urea, glucose, electrolytes, and other compounds were measured. During sleep deprivation, unrinary urea rose markedly, glucose decreased, and urinary electrolytes decreased. In contrast, urinary cortisol decreased only slightly. These findings indicate, that given ad libitum access to food and water, sleep deprivation per se can cause disturbances in normal metabolism.

Data from a study of the role of physical fitness in sustaining cognitive performance was reanalyzed and presented.

AVAILABLE

A study of the performance of tank crews in sustained operations in a simulated chemical environment (MOPP II and MOPP IV) in tanks (M60A3, M1, and M1A1) was conducted over the summer of 1984 in collaboration with the U.S. Army Armor and Engineer Board. This study, conducted at Ft. Knox, was entitled "Physiological and Psychological Effects of Nuclear, Biological, and Chemical Environments on Crews" (P^2NBC^2) was conducted in three phases: Phase I - turret trainers, Phase II - stationary tanks, Phase III - maneuvering tanks. In Phases II and III, crews in the M1A1 tanks (equiped with over-pressure and a micro-climate cooling system) were in MOPP II, while crews in the M60A3s and the M1s were in MOPP IV. Departmental personnel participated in all three phases of this study, including Phase III in the field. They collected data on crew performance using the department's performance assessment battery (PAB) and the affect/activation scale (AAS). Preliminary analysis indicates that M1A1 crews in MOPP II sustained higher levels of performance for longer periods of time than crews in the M60A3 and the M1 in MOPP IV.

A study in collaboration with the USAARL was conducted at Ft. Rucker on the performance effects of heat stress in helicopter pilots. Seven pilots tested with the PAB before and after three flights each while wearing MOPP IV gear. The study has been completed, and analysis of the PAB data is underway.

A study in collaboration with the U.S. Air Force was conducted of the effects of sickle cell trait on cognitive performance at high altitude. Thirteen pairs of subjects consisting of one control and one sickle cell trait pilot were tested on the department's performance assessment battery in a hypobaric chamber at simulated altitudes ranging from 5,000 to 25,000 feet. The results are currently being analyzed. Similar collaborative studies were conducted by the department to test the effects of anti-histaminic drugs on pilot performance at varying altitudes.

The complex demodulation program used in the analysis of circadian and ultradian rhythm data was modified to allow the delineation of overall underlying trends in a sequentially sampled data. This modification provides information as to the overall nature of the data set and allows the extraction of the rhythmic components unconfounded by other sources of variance inherent in the data or in its means of collection. Also, the filter used in signal processing within the complex demodulation program was manipulated resulting in the exact apportionment of variance for each frequency component in a time series and therefore providing statistical values of relative contribution. Both enhancements have been incorporated into the current version of complex demodulation and are being utilized in the ongoing analysis of data from the 72 hour sleep deprivation study.

With regard to future work, a human research protocol was written to examine the recuperative effects of naps on cognitive performance, mood state, and selected sleep parameters across varying stress conditions during sustained operations. This protocol has passed scientific review and is currently under consideration by the human use committee.

Publications:

1. Babkoff, H., S.G. Genser, H. Sing, D. Thorne, S. Taube, D. Redmond, and F. Hegge The effects of sleep loss and modified continuous

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performance on psychological and performance variables I: Task description and dynamic changes in duty cycle. Technical Report, submitted to Div NP, WRAIR, 1984.

2. Kant, G.J., S.G. Genser D.R. Thorne, J.L. Pfalser, and E.H. Mougey Effects of 72 hour sleep deprivation on Urinary Cortisol and indices of metabolism. Sleep 7(1984)142-146.

3. Sing, H.C., S.G. Genser, H. Babkoff, D.R. Thorne, and F.W. Hegge Complex demodulation - A technique for assessing periodic components in sequentially sampled data. ARO Report 84-2: Proceedings of the Twenty-Ninth Conference on the Experiments in Army Research Development and Testing.

Presentations:

1. Pleban, R.J., D.A. Thomas, and H.L. Thompson Physical fitness as a moderator of cognitive work capacity and fatigue onset under sustained combat-like operations. Paper presented at the Sustained Operations Research Meeting, Ontario, Canada August 1984.

2. Sing, H.C., S.G. Genser, H. Babkoff, D.R. Thorne, and F.W. Hegge Complex demodulation - A technique for assessing periodic components in sequentially sampled data. Paper presented at the Twenty-Ninth Conference on the Experiments in Army Research Development and Testing.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION ~~DA OT~~ 6452
2. DATE OF SUMMARY 84 10 01
REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01
4. KIND OF SUMMARY D. Change
5. SUMMARY SCTY U
6. WORK SECURITY U
7. REGRADING
8. DISB'N INSTR'N CX
9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62777A	3E162777A879	AB	044 WWJ7
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 82/83-6.2/2			

11. TITLE (Precede with Security Classification Code)
(U) Neuroendocrine Response to Military Stress

12. SUBJECT AREAS
0619 Stress Physiology 0615 Pharmacology 0601 Biochemistry

13. START DATE 76 07
14. ESTIMATED COMPLETION DATE CONT
15. FUNDING ORGANIZATION DA
16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	4.0	589
85	4.0	600

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
Top, F H, Jr
d. TELEPHONE NUMBER (include area code)
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS
Division of Neuropsychiatry
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
Meyerhoff, J L
d. TELEPHONE NUMBER (include area code)
(202)-576-3559
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
Kant, G J
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
Oleshansky, M A

21. GENERAL USE
F I N A
MILITARY/CIVILIAN APPLICATION H

22. KEYWORDS (Precede EACH with Security Classification Code) (U)Stress;(U)Hormones:(U)Neuropeptides:(U)Combat Psychiatry; (U)Volunteers;(U)Lab Animals;(U)Rats;(U)RAM III

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To examine neuroendocrine correlates of stressors specific to the military environment. Types of stress to be studied will include physical and psychological stressors, including continuous performance and stressful social interaction.

24. (U) In both laboratory and field studies, we will attempt to correlate endocrine and performance data. This will provide a basis for optimization of work/rest schedules and stress management, and consideration of medical prevention/treatment regimens.

25. (U) 83 10 - 84 09. Urinary cortisol and electrolytes were measured in soldiers conducting two sustained-operations field exercises, each lasting 72 hours. Urine specific gravity and cortisol excretion increased markedly during both field exercises, while urine volume significantly decreased. The increase in cortisol excretion occurred primarily at night and disrupted the normal circadian variation in cortisol excretion. Two major clinical psychoendocrine protocols have been prepared. A study of psychoendocrine responses in persons undergoing treadmill testing in the Army's "over 40" program is yielding data showing rises in plasma beta-endorphin, ACTH and cyclic AMP during maximal exercise. A protocol is in review proposing study of psychoendocrine responses to stress in military personnel during recognition boards. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83 - 30 Sep 84.

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EDITION OF MAR 68 IS OBSOLETE

Project 3E162777A879 MEDICAL FACTORS ENHANCING SOLDIER EFFECTIVENESS

Work Unit: 044 Neuroendocrine Response to Military Stress

Investigators:
Principal: Meyerhoff, J.L., M.D.
Associate: Oleshansky, M.A., M.D., MC; Kant, G.J., Ph.D.; Belenky, G.L., M.D., LTC, MC; Mougey, E.H., M.S.; Pennington, L.L., B.S.

Objectives:

Neuroendocrine responses to stressors typical of combat and the military environment are studied in order to identify conditions and processes leading to physical and/or psychiatric breakdown in combat. Types of stressors studied will include physical and pharmacological as well as psychological stressors. In both laboratory and field studies, we will attempt to correlate endocrine and performance data. This will provide a basis for optimization of work/rest schedules and stress management, and consideration of medical screening, prevention and treatment regimens.

Progress:

During the last year, we assayed urine samples collected during a sustained field exercise conducted in April 1983. The field exercise consisted of a pretrial phase, a 3 day sustained operations phase which included tactical offensive and defensive maneuvers conducted in normal field gear, an intertrial interlude, a second 3 day field exercise in which the soldiers wore MOPP gear, and a recovery interval. Consecutive 12 hour urine samples were collected, urine volume measured and specific gravity determined. Urine aliquots were frozen and shipped to WRAIR for later analyses. Urine specific gravity increased and urine volume decreased during both 3 day field phases as compared to pretrial, intertrial and posttrial phases.

Urinary cortisol was measured because (1) it is responsive to stress in humans (2) it normally varies in a regular rhythm over the course of 24 hours and might prove to be a sensitive marker for sleep schedule disruption during a sustained field operation (3) it has significant effects on physiological function, e.g. protein and carbohydrate metabolism. Cortisol excretion varied markedly over the the different phases of the exercise. Significantly more cortisol was excreted during the two field phases. In addition the daily pattern of cortisol was affected by the round-the-clock sustained operations. In the pretrial period three times as much cortisol was excreted during the 0500 to 1700 (day) sample as during the 1700 to 0500 (night) sample. During the field trials, a larger proportion of the cortisol excreted was found in the night collection. Most of the additional cortisol excreted during the field phases was due to the additional nighttime excretion of cortisol.

Urinary electrolytes were determined since adequate hydration is a concern during extended operations especially those conducted in MOPP gear which might be expected to increase temperature and increase the difficulty of drinking (with gas mask on). Urinary potassium levels were somewhat increased during the two field phases. Urinary sodium levels were lowest during the field trial conducted in MOPP, possibly as a result of increased sodium loss via sweating rather than through urine excretion. However this effect was not statistically significant.

As reported last year, a study of 72 hr of sleep deprivation in human volunteers showed significant changes in urinary metabolites during the sleep deprivation. This year a report of these findings was published (see below). In addition, we have made significant progress in setting up a rat model of sleep disruption and extended operations (see Annual Report for Project 3M161102BS10, Work Unit 215, Sept., 1984). Continued studies with this model should allow comparisons with the human sleep deprivation study and facilitate the testing of potential useful interventions, e.g. changes in food type or use of pharmacological agents to improve performance.

In order to characterize the neuroendocrine responses to stressors typical of the military environment, we have begun a comprehensive study of the effects of physical and psychological tasks on a battery of neuroendrocrine and neurochemical indices felt to be markers of exertion and/or stress. We are attempting to profile and compare the biomedical responses to physical activity to the biomedical responses seen in conjunction with the psychological stress associated with performance demands such as Soldier of the Month Boards.

To determine the effect of physical activity on biomedical responses, we have begun an approved study at Walter Reed Army Hospital in conjuction with the Dept. of Cardiology on neuroendocrine responses to treadmill testing in the "Over 40" military population. We are gathering data on plasma prolactin, cortisol, beta-endorphin, ACTH, cyclic nucleotides and catecholamine responses to treadmill testing in control subjects with various levels of physical conditioning. This control data is being compared with findings in subjects who are undergoing repeat treadmill testing for an abnormal treadmill test (significant ST depression on EKG suggestive of cardiac ischemia). As these subjects do not report physical symptoms of cardiac ischemia such as angina but are found to have evidence of significant cardiac disease on this "Over 40" screening exam, they represent a potentially very interesting population for the study of neuroendocrine responses to physical exertion. These subjects generally will have had a cardiac catheterization to determine the extent of coronary artery disease prior to the repeat treadmill test which will allow us to determine false positive responders, i.e. those subjects with no gross

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coronary artery disease but an abnormal EKG on treadmill testing.

To date, we have collected plasma samples from ten subjects and have begun assaying these samples for the various parameters listed above. We can already report a significant increase of beta-endorphin, beta-lipotrophin, ACTH, and cyclic AMP to the exertion of treadmill testing in most subjects. In those subjects who have a vigorous response, it appears that some show a rapid decline of these indices to baseline values after stopping running while others do not. We are currently attempting to see if this correlates with age or prior conditioning. Similarily, some subjects show no elevation of beta-endorphin or ACTH with activity which may correlate with lack of prior physical conditioning. Additionally, we have demonstrated that plasma catecholamines are elevated with physical activity but more significantly increase several fold upon the completion of exercise. This is a very dramatic effect which has only recently been appreciated in the exercise literature but may have far reaching implications in regards to sudden death after physical activities such as marathon running, etc.

We currently have a protocol pending approval to undertake a study of stress responses associated with performance demands of a psychological nature. This will be a WRAMC wide study of enlisted soldiers participating in recognition boards. We will be examining the plasma neuroendocrine parameters listed above for treadmill study as well as urinary hormones, heart rate responses and a battery of psychological scales to determine the relationship of both psychological and physiological stress responses to psychological make-up. This study should complement and extend the treadmill study by providing important information on biomedical and psychological responses to stressors of a non-physical nature. Taken together, these two studies should allow us to profile the biomedical responses to stress and determine the most sensitive and/or useful indices for developing future studies in this field.

It should be noted that these studies require extremely sensitive assays for the measurement of plasma hormones and catecholamines. We have developed an HPLC assay for catecholamines which is able to measure resting levels of these compounds in less than one milliliter of plasma. A radioimmunoassay for human beta-endorphin has been developed for use in assaying plasma samples from the human exercise study. Used in combination with an extraction and separation procedure developed in this laboratory, we are now able to measure separately both beta-endorphin and beta-lipotrophin in 1 ml of human plasma. Our data collected thus far are showing an increase in both hormones during prolonged exercise (see above). We are also using a radioimmunoassay for measuring plasma ACTH in these same samples.

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Future Objectives:

In conjunction with the Dept. of Behavioral Biology, we plan to continue to study the biochemical correlates of the performance decrements induced by sleep deprivation. In particular, we wish to determine whether additional food intake might prevent the increase in urea excretion seen in the initial study. We plan to begin neuroendocrine studies of soldiers participating in soldier recognition boards pending institutional approval. After establishing recognition boards as a clinical stress model, we plan to initiate intervention studies such as behavioral desensitization or use of beta-adrenergic blocking drugs. In collaborations with Duke University and the Massachusetts General Hospital, additional studies are planned on the effects of stress on subjects with "type A" personalities, the stress of public speaking, and stressful interviews. We also plan studying patients with "panic disorder", a condition with marked similarities to "Soldier's Heart".

Presentations:

1. Meyerhoff, J.L. Dept. of Pharmacology, Duke University School of Medicine, Durham, NC, Jan 1984. Invited Lecture: "Adrenalectomy Blocks the Stress-Induced Increase in Pituitary cyclic AMP".

2. Meyerhoff, J.L. Dept. of Biochemistry, George Washington University, Wash., D.C., June 1984. Invited Lecture: "Biochemical Assessment of Stress in vivo".

3. Chernow, B., Geelhoed, G., Reed, L., Beardsley, D., Holt, M.R., Burman, K.D., Holaday, J.W., Meyerhoff, J.L. and Lake, C.R. Glucagon as a Chronotropic Agent. National Society for Critical Care Medicine, San Francisco, CA, 21 May, 1984.

4. Jarrard, L.E., Levy, A., Meyerhoff, J.L. and Kant, G.J. Intracerebral injections of AF64A: An animal model of Alzheimer's Disease? New York Academy of Sciences Conference on Memory Disfunction, 13-15 June, 1984.

5. Meyerhoff, J.L. Dept. of Neuropathology, Armed Forces Institute of Pathology, Wash., D.C., Aug., 1984. Invited Lecture: "Neurochemical and Neuropharmacological Studies in the Kindling Model of Post-Traumatic Epilepsy".

Publications:

1. Kant, G.J. Lenox, R.H., Bunnell, B.N., Mougey, E.H., Pennington, L.L., and Meyerhoff, J.L. Comparison of stress response in male and female rats: pituitary cyclic AMP and plasma prolactin, growth hormone and corticosterone. 1983. Psychoneuroendocrinology 8, 421-428.

2. Bunnell, B.N., Hills, W.E., Terrell, D., Tigges, J.H., Mougey, E.H., Pennington, L.L. and Meyerhoff, J.L. Plasma Beta-Endorphin and Beta-Lipotropin Response to Conditioned Stimuli. 1983. Neuroscience Abstracts 9, 1124.

3. Belenky, G.L., Gelinas-Sorrell, Kenner, JR. and Holaday, J.W. Evidence for delta receptor involvement in the post-ictal antonocioceptive responses to electroconvulsive shock in rats. 1983. Life Sciences 33: Supplement I, 583-585.

4. Emurian, H.H., Brady, J.V., Meyerhoff, J.L. and Mougey, E.H. Small groups in programmed environments: Behavioral and biological interactions. 1983. The Pavlovian Journal of Biological Science. 18: 199-210.

5. Oleshansky, M.A., Reisberg, B., and Ferris. S.H. Serum Dopamine-beta-Hydroxylase in Alzheimer Type Dementia. Society for Neuroscience Abstracts, 13th Annual Meeting, Boston, Mass., Nov. 6-11, 1983, 9:1, 97.

6. Belenky, G.L. Training in military and combat psychiatry. In O. Adejala and F.D. Jones (Eds.) War and its Aftermath, John West Publishers, Lagos, 1983, 196-202.

7. Kaufman, L.W. and Belenky, G.L. Staying Alive: knowing what to do until the medic arrives. 1984. Military Review. 64: 28-33.

8. Emurian, H.H., Brady, J.V., Ray, R.L., Meyerhoff, J.L. and Mougey, E.H. Experimental Analysis of Team Performance. Naval Research Reviews. 1984. U.S. Govt. Printing Office, Wash. D.C., Vol. XXXVI:3-19.

9. Kant, G.J., Genser, S., Thorne, D., Pfalser, J., Mougey, E.H. Effects of 72 hr sleep deprivation on urinary cortisol and indices of metabolism. Sleep 7, 142-146, 1984.

10. Oleshansky, M.A., Greenspan, D.L., Reisberg, B., and Ferris. S.H. Serum Dopamine-beta-Hydroxylase and Primary Degenerative Dementia. 1984. American Psychiatric Assocation 137th Annual Meeting, CME Syllabus and Scientific Proceedings, p. 244.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY		1. AGENCY ACCESSION DA 305982	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. NEW	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING
8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT			

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62777A	3E162777A879	AB	045 WWJB
b. CONTRIBUTING				
~~c. CONTRIBUTING~~ XXXXXXXXXXX	STOG 82/83-6.2/2			

11. TITLE *(Precede with Security Classification Code)*
(U) Military Stress: Biomedical Measurement Research

12. SUBJECT AREAS
0510 Psychology, 0616 Physiology, 0619 Stress Physiology

13. START DATE 84 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		84	0.0	00
c. TYPE	d. AMOUNT	85	3.0	872
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Neuropsychiatry
b. ADDRESS *(include zip code)* Washington, D.C. 20307-5100	b. ADDRESS Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR REDMOND, D P
d. TELEPHONE NUMBER *(include area code)* 202 - 576-3551	d. TELEPHONE NUMBER *(include area code)* 301 - 427-5521
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* TAUBE, S L g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* THORNE, D R

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Electrophysiology; (U) Psychophysiology; (U) Psychophysics; (U) Stress; (U) Performance; (U) Volunteers (U) RAM III

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Design, develop, evaluate and deploy non-invasive psychophysiologic monitoring systems as technology base for laboratory and field studies of stress resistance and performance in military environments.

24. (U) New technologies in signal acquisition and processing are studied and applied through in-house and extramural efforts emphasizing temperature, sleep/activity, cognitive performance, neuropsychological and cardiovascular function, and rhythmic interactions among these measures. Basic research is coupled with design and development to achieve sophisticated research tools suitable for monitoring health and performance of active soldiers.

25. (U) 83 10 - 84 09. This work unit supplants Agency Accession DAOC 6473. Non-invasive Monitoring of Health Performance. The program is undergoing a transition from the successful 6.1 basic research and development of non-invasive psychophysiologic monitoring, to one of applied 6.2 research, emphasizing deployable applications of the instrumentation in Army field tests, large scale training exercises, and other soldier performance measurement studies. For technical progress report on the 6.1 research which preceeded this work, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION DA OC 6470	2. DATE OF SUMMARY 84 10 01	*REPORT CONTROL SYMBOL* *DD-DR&E(AR) 636*				
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62777A	3E162777A879	AA	046 WWQ1
b. CONTRIBUTING				
c. [illegible]	STOG 82/83-6.2/2			

11. TITLE *(Precede with Security Classification Code)*
(U) Medical Factors Limiting Soldier Effectiveness

12. SUBJECT AREAS
0619 Stress Psychology 0510 Psychology

13. START DATE: 7710

14. ESTIMATED COMPLETION DATE: Cont'

15. FUNDING ORGANIZATION: DA

16. PERFORMANCE METHOD: C. In-house

17. CONTRACT/GRANT

a. DATE EFFECTIVE

EXPIRATION

b. CONTRACT/GRANT NUMBER

c. TYPE

d. AMOUNT

e. KIND OF AWARD

f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	3.0	75
85	3.0	60

19. RESPONSIBLE DOD ORGANIZATION

a. NAME: Walter Reed Army Institute of Research

b. ADDRESS *(include zip code)*: WASH, D.C. 20307 5100

c. NAME OF RESPONSIBLE INDIVIDUAL: TOP, F H JR

d. TELEPHONE NUMBER *(include area code)*: (202) 576-3551

20. PERFORMING ORGANIZATION

a. NAME
Walter Reed Army Institute of Research
US Army Medical Research Unit-Europe

b. ADDRESS
HQ 7th Medical Command
APO New York 09102

c. NAME OF PRINCIPAL INVESTIGATOR: VAN VRANKEN, E

d. TELEPHONE NUMBER *(include area code)*

f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*: ROCK, S

g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*: KAUFMAN, L

21. GENERAL USE: FINA

MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Epidemiology; (U) Stress; (U) Psychiatry; (U) Volunteers; (U) Soldier Effectiveness; (U) RAM III

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) To identify factors in the military organizational, social, psychological, and physiological environment that create or increase risk for psychiatric breakdown, behavioral dysfunction, psychosomatic and physical illness, all of which impact on individual and unit effectiveness and consume health care resources.

24. (U) The methods of epidemiology, including records analysis, population and demographic analysis, questionnaires, field and cohort studies, and various observation methods are employed to develop requisite data.

25. (U) 83 10-84 09 A study of the role of stress problems in patient admissions is complete. Preliminary results show that patients admitted for stress are experiencing problems on the job, with their supervisors or at home in their family life. A study of the expectations of soldiers assigned to Europe, and the health implications of those expectations, is in progress. A continuation of the integration and socialization of officers in USAREUR, including combat support and combat service support officers, is in progress. Interest in the article on first aid training for soldiers (Military Review, 1984) has led to two new projects: 1) a survey of first aid training in other armies, and 2) planning for a study which will assess morale and attitudes of medics in Europe as well as the attitudes of soldiers toward the medics. Data has been collected and analysis begun on a study of factors which influence gunnery performance of tank crews. Report on problems associated with Noncombatant Evacuation is in draft form. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

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PROJECT 3E162777A879 MEDICAL FACTORS ENHANCING SOLDIERS EFFECTIVENESS

Work Unit 046 Medical Factors Limiting Soldier Effectiveness

Investigators

Principal: Van Vranken, LTC E.W.
Associates: Rock, CPT S.K., Jr.
Kaufman, CPT L.W.

Description

This field unit, stationed in West Germany with the U.S. Army Europe and Seventh Army, identifies and investigates physical, psychological, social, and organizational factors bearing on the individual, unit performance, and battle readiness. Current efforts focus on seven areas identified by commanders as important concerns within the European theater.

REFORGER STRESS CASUALTY STUDY (1983)

Data analyses for the REFORGER stress casualty study have been completed, and the written report is available in draft form. Several hypotheses about stress reactions have been tested during REFORGER. Since REFORGER is more stressful than "normal" duty, one hypothesis was that proportionately more soldiers would be hospitalized for stress during REFORGER than at other times during the year. This hypothesis, however, was not supported. Of the patients hospitalized during the exercise at least 15% were for stress-related problems. The data indicate that soldiers deployed from the United States have higher scores on cohesion and well-being measures and lower scores on psychological stress than individuals deployed within Germany. Further, female SM show lower cohesion scores and higher stress scores than male SM, although this finding needs further investigation. In general, soldiers with high cohesion scores have significantly less stress and score higher on the well-being scale than those with low cohesion scores. Soldiers assigned to combat arms units have significantly better cohesion scores than those assigned to combat support or combat service support units, but did not show differences in well-being or stress.

SOLDIERS EXPECTATIONS STUDY

Initial data collection for the soldiers expectations study has been completed. Data were collected from over 400 soldiers who had just arrived in Germany and were in-processing through the 21st Replacement Battalion at Rhein-Main AFB in Frankfurt. Eighty-four percent of soldiers participating in the questionnaire and interview process were between grades E1 and E4. Preliminary analyses show that about 44% of personnel reporting to Germany are volunteers. Only 18% of those interviewed are unhappy with the overseas assignment. Most of the soldiers report only limited control over what happens to them in the military. In general, most of the soldiers have a favorable attitude towards Germany, its people and traditions, and expect to enjoy and take advantage of recreational opportunities here and in other European countries. These soldiers are looking forward to joining their new units, and believe the work will offer new challenges and experiences. They also believe that more will be expected of them in the overseas unit than stateside. A potential problem is that these, and other high expectations, may not be met,resulting in frustration, development of negative attitudes, and poor job performance in the soldier. Phase Two of this study will include a followup on these soldiers approximately 6 months after their arrival date in Germany.

COMPANY GRADE OFFICER SOCIALIZATION STUDY

The company grade officer socialization study is a continuation of earlier studies on the socialization of lieutenants and company commanders into their units. There were several areas about which company commanders had expressed some confusion or question concerning their own roles and duties. These areas concerned the importance of lieutenants to the functioning of the company, training leadership in the lieutenant, how the company commander learns to train lieutenants, differences between captains and lieutenants in the officer ethic, and who trains the company commander to do his job. The present study is in the planning stages, and will include officers from combat support and combat service support units, as well as female officers. These officers were not included in the initial studies.

FIRST AID TRAINING

The publication of the article by Kaufman and Belenky, "Staying Alive: Knowing What To Do Until the Medic Arrives" (Military Review, January 1984), generated a positive response in the military community and has resulted in increased emphasis on first-aid training for soldiers in combat units in Europe. It also generated a demand for more detailed information on the training done in other modern armies. An effort is currently being made to collect information on first-aid training in other armies, including the

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Israeli Defense Force, the British Army, and the West German Bundeswehr. This information is to be combined with other, recently acquired information on the value of first-aid training, and put in a written report.

MORALE, COHESION AND ATTITUDES AMONG AND TOWARD COMBAT MEDICS

Studies on morale and cohesion in the military have concentrated almost exclusively on combat arms units. Evidence suggests, however, that morale and cohesion factors are just as important in the performance of support units as they are in combat units. The Israeli Defense Force, for example, reports higher rates of stress breakdown in support units than in combat units. A study, currently in the planning stages, will attempt to measure morale and cohesion among U.S. Army medics stationed in Europe. The study will also assess the attitudes of soldiers in line units on their relationship and interaction with medics.

NONCOMBATANT EVACUATION

Data collected on the attitudes of members of the military communities in Europe toward the Noncombatant Evacuation Operation (NEO) are being used as the basis for a paper which outlines some of the problems with the current NEO plan. Recommendations on how to improve the plan through a deemphasis on immediate evacuation and an increased emphasis on sense of community and community self-reliance will be addressed. This paper is currently in draft form and, pending revision, will be submitted for clearance.

TANK CREW GUNNERY

The purpose of this research is to determine the relationship of selected demographic and psychosocial factors in crew structure to tank crew performance. Data were obtained from 45 tank crews of an armor battalion participating in their annual gunnery qualification at Grafenwoehr. The data on gunnery performance has been collected and will be correlated with demographic and psychosocial data collected on individual crew members. Analyses are in progress.

NEW MANNING SYSTEM (COHORT) LONGITUDINAL STUDY

In concert with the Department of Military Psychiatry, Division of Neuropsychiatry, WRAIR, this unit participates in the longitudinal study of family and unit issues of COHORT units presently assigned to USAREUR. Using both individual and group interview techniques, a series of core issues are addressed, including unit cohesion, cross rank relationships, combat readiness and effectiveness, leader/soldier relationships, as well as family adjustment and family/unit relationships.

This study follows a sample of company-sized COHORT units from their inception to their dissolution in a three-year timeframe. Initial findings suggest that COHORT units are perceived as more cohesive and better horizontally bonded than conventional units. COHORT units are also perceived as probably having longer staying power in combat and better able to resist the initial disruption and shock of combat than the average conventional unit.

Family issues are also assessed. A limited number of units have successfully implemented family programs. These successes have in common: 1) Taking time to insure that families are adequately settled after a major move, and 2) Getting families together very early and informing them of community resources, outlining husbands' duties, and sponsoring unit/family activities. Such gatherings provide an excellent opportunity for wives to begin to develop supportive relationships.

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a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
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c. TYPE	d. AMOUNT			
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a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Division of Neuropsychiatry Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL Top, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR Holaday, J W
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (202)-576-3028
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	g. NAME OF ASSOCIATE INVESTIGATOR (if available) Tortella, F C

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23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To develop neuropharmacological insights into the etiology and treatment of circulatory shock and trauma, and to evaluate electrophysiological, neuroanatomical, autonomic, endocrine and immunological correlates of performance and casualty management. The integrated efforts of a team of experts in these areas allows for a multi-faceted approach using in vitro and in vivo techniques to establish these interrelationships. Results will provide new pharmacological approaches to the management of military casualties as well as to performance enhancement.

24. (U) Measurements of physiological, pharmacological and behavioral function in animals will be accomplished with standard techniques, including the use of surgically catheterized rats with implanted brain electrodes. Physiological and behavioral performance will be assessed. Blood samples will be analyzed for concentrations of circulating hormones, neurotransmitters and non-cellular components of immune responses. Baseline measurements will be contrasted with responses to endogenous or exogenous agents to evaluate functional interactions in the above systems.

25. (U) 83 10 - 84 09. In models of circulatory shock, it was shown that the precipitous fall in arterial pressure was mediated inpart by anaphylatoxins and that injections of thyrotropin releasing hormone (TRH) reversed this action better than opioid antagonists. Tolerance to morphine exacerbated endotoxic shock, yet did not alter responses to the opioid antagonist naloxone. The gut peptides cholecystokinin and glucagon wereshown to have acute cardiovascular effects of their own and to have anti-opiate effects on analgesic and cardiovascular endpoints. A study was initiated to establish the role of gut-liberated endotoxins in hemorrhagic shock hypotension. The role of the adrenal cortical hormones in regulating blood-brain barrier was further documented. Several different methods indicated that multiple opioid receptors may be part of the same macromolecule. Anti-seizure effects of opiate peptides were established, and nerve growth factor was shown to alter development of cholinergic neuronal pathways pertinent to organophosphate toxicity. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 83-30 Sep 84.

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Project: 3E162777A8879 **MEDICAL FACTORS ENHANCING SOLDIER EFFECTIVENESS**

Work Unit 047 **Neuropharmacological Management of Military Performance and Casualties**

Investigators:
Principal: Holaday, J.W., Ph.D.
Associate: Mobley, W.C., MAJ, MC; Long, J.B., CPT, MS; Bernton, E.W., MAJ, MC; Tortella, F.C., Ph.D.; Malcolm, D.S., Ph.D.; Johnson, C.E.; Ruvio, B.A., M.S.; Robles, L.E., M.S.; Rogers, O., B.S.; Eschevarria, E., M.S.; Encarnacion, E.M., B.S.

Objectives:

To develop experimental insights into the etiology and treatment of circulatory shock and trauma; to evaluate the role of endogenous substances in arousal and depressed states; and to provide pharmacological insights into the mechanisms of adaptation and habituation to environmental and behavioral stressors. This coordinated effort by a team of experts will allow for a multifaceted, simultaneous evaluation of neuropharmacological, electrophysiological, biochemical, developmental, anatomical, endocrine and immunologic correlates of research in these areas.

Over the last year, studies have characterized specific molecular interactions among multiple opioid receptors involved in shock pathophysiology and chemically induced convulsions. In order to elucidate neurobiological and immunological mechanisms in circulatory shock, convulsive behaviors and nervous system development, experiments have been performed using surgically catheterized rats as well as rats implanted with brain catheters and electrodes. Physiological function and behavioral responses to drugs were assessed. Blood and tissue samples were evaluated for changes in receptor binding characteristics, alterations of circulating hormones and changes in cellular and non-cellular components of the immune system. New immunoassay procedures are being developed to increase the sensitivity and selectivity of assay systems. In all experiments, baseline measurements were contrasted with the effects of endogenous or exogenous agents in order to quantify results. Additionally, injected radioisotopes were used to evaluate potential mechanisms involved in the transportation of hormones and drugs into the central nervous system.

The ultimate objective of these studies is to develop a greater understanding of the physiological and pathophysiological responses to shock, trauma, and altered behavioral states in order to establish new pharmacological approaches to biomedical problems of clinical and military relevance. As a result of these investigations, it is hoped that defining the etiology of these disorders will allow for more selective pharmacological therapies.

Progress:

I. Management of shock and trauma:

The potential role of the complement system in mediating the rapid hypotensive effects of endotoxic shock was evaluated using cobra venom factor (CVF). This substance acutely generates the anaphylatoxins C3a and C5a by a specific activation of the complement cascade. These anaphylatoxins are known to contribute to anaphylactic shock hypotension, presumably by increasing vascular permeability. Several hours after a single CVF injection, the complement system becomes depleted, rendering animals unresponsive to subsequent CVF injections. Lipopolysaccharide endotoxins from gram negative organisms also activate the complement cascade (via the alternative pathway). To determine if anaphylatoxins mediate the acute depressor effects of endotoxic shock, the cardiovascular effects of endotoxin were evaluated in rats depleted of complement by prior CVF administration. Indeed, it was shown that CVF pretreatment 24 hrs earlier prevented the usual immediate (1-10 min) hypotensive response to endotoxin, however the later depressor effect (that occurs at 45 min) was unaffected by complement depletion.

From our prior work, we knew that the acute hypotensive response to endotoxins is reversed by the specific opioid antagonist, naloxone. To address the possible functional relationships between endogenous opioid systems and the complement system, naloxone was injected immediately following the onset on CVF-induced (anaphylatoxin) hypotension. Naloxone partially reversed this hypotensive action of CVF, indicating functional interactions between anaphylatoxin hypotension and endogenous opioid-induced hypotension. The precise level of this interaction remains for future study.

As another approach to the reversal of anaphylatoxin hypotension, we injected thyrotropin releasing hormone (TRH), a peptide molecule that we have previously demonstrated to be even more efficacious than naloxone in reversing the pathophysiological effects of certain forms of circulatory shock and spinal trauma. Using CVF as a model of anaphylactic shock, the utility of TRH in treating this form of hypotension was established.

An important consideration in treating circulatory shock or spinal cord trauma with opioid antagonists involves their use in patients that have been previously exposed to narcotic drugs. For example, the opioid antagonist naloxone will precipitate acute withdrawal responses in morphine or heroin dependent individuals. How does this affect the therapeutic responses to naloxone when used for the treatment of shock and trauma? To address these issues experimentally, we asked two questions: Does opiate tolerance modify the hemodynamic response of rats exposed to endotoxic shock? Does naloxone improve or worsen the shock state in opioid-tolerant animals?

Studies showed that morphine tolerance enhanced susceptibility of conscious rats to endotoxic shock and markedly increased the pressor response to naloxone following endotoxic shock. Naloxone also precipitated acute opiate withdrawal in the tolerant rats while reversing the hypotensive effects of endotoxin. This was associated with a 25 mmHg increase in blood pressure beyond the effect of naloxone in non-tolerant rats subjected to endotoxemia. Thus, the pressor response to naloxone in tolerant, endotoxemic rats is greater than the sum of the pressor responses of opiate tolerant or of endotoxemic rats given naloxone. These results have obvious clinical implications regarding the safety and efficacy of naloxone in the treatment of narcotic tolerant patients in shock, and also suggests narcotic tolerance may enhance the centrally-mediated, naloxone sensitive hemodynamic component of acute endotoxic shock. Thus, prior exposure to narcotic analgesics enhances the probability of shock, yet is not a deterrent for the use of opioid antagonists in its treatment.

II. Multiple opioid receptor interactions - Functional implications:

Since general opioid antagonists such as naloxone block the pain-relieving effects of opioids while exerting therapeutic effects in circulatory shock, a selective approach was sought to allow for shock reversal while still enabling pain relief from endogenous opioids or morphine. Using the selective δ antagonists ICI 154,129 and ICI 174,864, as well as the μ antagonists β-funaltrexamine and naloxazone, it was shown that the "opioid" component of shock pathophysiology is mediated by the release of endogenous opioid peptides which act upon δ receptors, whereas the analgesic properties of injected morphine are mediated at μ receptors. This selectivity of action at distinct subpopulations of opioid receptors allows for the potential use of δ opioid antagonists to reverse shock, while the μ-agonist morphine could be co-administered to allow for relief of the traumatic pain which may accompany severe injury.

Over the past year, we have continued these studies of opioid receptor pharmacology to incorporate an assessment of the potential role of κ receptors in these interactions. It was demonstrated that many of the opioid molecules that are classified as κ agonists (including dynorphin) also interact with μ and δ receptors in a unique manner. Specifically, we have demonstrated that κ agonists are μ antagonists and antagonists of δ antagonists. Since it may be argued that cardiovascular responses in endotoxic shock are far removed from initiating events at the receptor, we tested these drugs in models of chemically induced seizures that also respond to opiates. Using the volatile convulsant, flurothyl, the anticonvulsant properties of μ and δ agonists were tested in the presence of selective μ and δ antagonists as well as compounds formerly classified as κ agonists. Once again, it was demonstrated that κ agonists are μ antagonists and antagonists of δ antagonists. This precise pattern of molecular interactions of opioid agonists and antagonists using the entirely separate pharmacological endpoints (endotoxic shock and chemical seizures) forces a reconsideration of receptor dynamics. Instead of the classic assumption that these three "receptors" are distinct, unrelated entities, our observations indicate that κ, μ and δ binding sites may functionally interact as part of the same macromolecular complex.

III. Cholecystokinin and glucagon-Neuropeptides with autonomic effects:

Neuropeptides, such as the endogenous opioids or TRH, have been shown by us to have potent and long-lasting autonomic and behavioral effects that predict their use or the use of selective antagonists of their actions as new treatments for a number of biomedical disorders. Two other neuropeptides, cholecystokinin-octapeptide (CCK-8) and glucagon, are found both in the brain and the gastrointestinal system. Although their role in digestive processes has been known for decades, as yet little is known about their function in the brain. Recently it has been shown that CCK-8 has anti-opioid properties. Because of our considerable interest in the utility of anti-opioid compounds in the treatment of shock and trauma, we investigated interactions between opioid systems as well as the direct effect of these neuropeptides on autonomic function.

Results from initial studies have shown that both central and peripheral administration of CCK-8 attenuates morphine-induced analgesia as measured by the hot plate and tail flick tests. Additionally, CCK-8 (iv) caused a dose-related, transient hypertension lasting from 1-5 min followed by a return to baseline. In these same animals, CCK-8 also produced an initial bradycardia which was followed by a more persistent tachycardia at higher doses. From preliminary studies, it appears that CCK-8 (10-100 micrograms/kg, iv) may attenuate the acute hypotension following endotoxic shock in a dose-related manner. These preliminary studies will be extended to include other models of shock such as hemorrhage and spinal cord ischemia. The mechanisms underlying the interactions between CCK-8 and opioid systems are presently being investigated.

Glucagon has been used clinically to treat β-blocker overdosage in a few preliminary trials. β-blocking adrenergic antagonists are finding increasing usage for numerous disorders, including hypertension, angina, migrane, familial tremors, etc., and they may have potential as antianxiety drugs that are useful in battlefield environments. Thus, the prior exposure to β-blocking drugs may not only complicate the use of other pharmacological agents, but may even block the use of β-agonists such as epinephrine that are classically used in critical care medicine. Since glucagon may have potent effects in overcoming the responses to β-blockers, as well as as

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yet undefined autonomic effects of its own, we studied autonomic responses to glucagon by itself and together with other drugs.

Glucagon by itself has a chronotropic effect on the heart that may be mediated by changes in calcium availability. However, this effect of glucagon upon heart rate is not further augmented by the addition of calcium. More recent studies have shown that glucagon administration attenuates the degree and duration of morphine-induced bradycardia and hypotension. Furthermore, results from preliminary studies suggest that this effect of glucagon is mediated within the brain rather than at peripheral sites.

IV. Electrophysiological correlates of performance and injury:

Changes in the excitability of the brain result from external causes such as trauma or drug administration as well as internal causes such as occur during seizures. Three fundamental approaches were developed to investigate the cause and effects of altered brain excitability. These included a continuation of our characterization of the effects of electroconvulsive shock (ECS), evaluation of chemically-induced seizures and measurements of electroencephalographic responses during sleep, wakefulness, or in response to drug injections.

An extensive EEG analysis of receptor-selective opioid peptide agonists and antagonists was initiated. Comprehensive dose-response, time-course and antagonist interaction studies showed that the transient nonconvulsive EEG seizures produced by opioid peptides is due to their selective, primary action at μ-receptors and not at the δ-opioid receptor as previously suggested. Following this initial CNS excitability produced by opioids, there is a subsequent period of prolonged CNS depression that appears quite similar to the EEG pattern observed following ECS seizures in rats. This EEG pattern may be consistent with an anticonvulsant interval following opioid administration or after a single seizure.

A rat model of intermittent maximal electroshock seizures was implemented which allowed us to study this phenomona of postseizure inhibition. Opioid substances are potent anticonvulsants, and endogenous opioids are released by ECS. The possibility that the release of endogenous opioids by seizures subsequently results in an endogenous anticonvulsant response was investigated. Results demonstrated that opioid antagonists such as naloxone blocked the endogenous anticonvulsant response following seizures. A novel experimental approach involving a cross-transfusion of cerebrospinal fluid (CSF) from rats exposed to seizures to naive rats was also used. It was shown that CSF obtained following seizures from donor rats decreased seizure severity and incidence in recipient rats. Additionally, CSF levels of the endogenous opioid β-endorphin were elevated, and it was shown that the opioids derived from the pituitary are important in decreasing the probability of recurrent seizures in this rat model. Thus, endogenous opioid systems play a critical physiological role in inhibiting the probability of repeated seizures immediately following the first seizure. These results may pertain to the endogenous mechanisms of seizure limitation during epilepsy.

We demonstrated that repeated daily ECS in rats, a model that may equate to the clinical use of repeated daily electroconvulsive therapy in depressed humans, results in a significant increase in the numbers of opioid receptors in the brain. These effects were similar to the effects of opioid tolerance on receptor numbers. As revealed by the use of sodium in the binding assays, both instances resulted in a conversion of the opioid receptors to the antagonist binding conformation. The precise functional significance of these observations remains for the future, however these studies extend our earlier observations that had demonstrated that ECS functionally activates endogenous opioid systems.

In the never-ending search for a better therapeutic antiepileptic agent, a potent anticonvulsant profile was demonstrated for a new opioid analgesic, U50,488, against ECS seizures, and not against chemically-induced seizures. Due to the possible non-addictive nature of this κ receptor analgesic (and its derivatives?), the potential development of a new, efficacious class of antiepileptics drugs for treating grand mal or psychomotor attacks must be considered.

V. Factors governing brain access of neuropharmacological substances:

The blood-brain barrier (BBB) maintains a constant extracellular environment for the nerve cells of the brain, protecting the central nervous system from changes in plasma composition, including peripherally released hormones as well as injected drugs or toxins. Research interest in the reciprocal interactions between the CNS and the periphery, coupled with recent evidence indicating that cerebral microvascular permeability may be responsive to neural and humoral influences, prompted our interest in potential roles of the endocrine systems, specifically the pituitary-adrenal axis, in maintaining homeostasis within the CNS through actions on the blood-brain barrier. To address this hypothesis, we examined the effects of adrenalectomy, selective adrenal demedullation and corticosterone replacement on the permeability of the blood-brain barrier to the macromolecule ^{125}I-bovine serum albumin. It was observed that total adrenalectomy, but not selective adrenal demedullation, significantly increased the permeability of ^{125}I-BSA into the brain, and that corticosterone replacement in adrenalectomized rats restored normal blood-brain barrier function. These results indicate that the pituitary-adrenal axis may permissively regulate the entry of macromolecules into the CNS, and may thereby indirectly alter the central actions of diffusion-limited drugs and humoral substances. Thus, stress-induced changes in adrenal function, or the administration of glucocorticoid drugs for various medical disorders, may modify the permeability of drugs and hormones into the brain.

VI. Neuroanatomical interactions with biological responses:

Nerve growth factor (NGF) is a peptide neuromodulator that has important trophic effects in the proliferation and synaptogenesis of peripheral autonomic neurons. We have continued experiments designed to establish the possible role of this substance in the development of brain neurotransmitter pathways. Previously, it was shown that NGF enhanced the development of cholinergic neuronal systems in the brain. Additionally, we have participated in immunohistochemical studies of the anatomical markers of neuronal degeneration in Alzheimers disease, namely the occurrence of neurites in plaques in an animal model of dementia using aged monkeys. Plaques have been shown to contain the neurites of several different neurotransmitter systems. This work is pertinent to an understanding of the etiology and treatment of the degenerative neurologic disorders of dementia, including Alzheimer's disease and neurotoxic effects of organophosphate inhibitors of cholinesterase activity.

We have extended our observations on the effect of NGF on basal forebrain cholinergic neurons. This has included evaluation of its effect on septial cholinergic neurons before and during synaptogenesis. Data indicate that hippacampal choline acetyltransferase (ChAT) activity may be regulated differently in growing neurites than in those which have already established synaptic contact. As another approach to the detection of NGF activity within the brain, we have initiated molecular-biological studies of NGF messenger RNA levels in brain tissue. This has involved the development of methods for measuring the rate of incorporation of radiolabelled amino acids into ChAT molecules in vivo. These latter studies have paved the way for determining whether NGF increases ChAT activity by increasing the number or the specific activity of ChAT molecules.

Over the past year, we have been successful in purification of NGF to homogeneity using a series of liquid chromatographic separations. The last of these has employed high performance reverse phase liquid chromatography. Studies are presently underway to characterize this NGF preparation for its effect in vitro & in vivo. A by-product of these studies has been the identification of a molecule which is physico-chemically quite similar to NGF. Preliminary data suggest that this is a modified form of NGF. Again, studies are underway to characterize its biological effects & its structural relationship to NGF.

As part of an effort to define the role(s) of the neuronotrophic substance NGF in central and peripheral neuronal function, we have initiated efforts directed to measurement of levels and changes in levels of NGF during development and following neuronal injury and intoxication. The technical requirements for such measurements are considerable, and to date, conventional assay techniques have proven to be inadequate.

Consequently, we are developing a novel enzyme-linked radioimmunoassay which theoretically should overcome many of the problems of sensitivity and specificity plaguing other assays. This system employs purified or monoclonal NGF antibodies linked to a solid phase for extraction of NGF from sample preparations. After washing, NGF bound by the immobilized antibodies is bound by a second series of NGF monoclonal antibodies from which a molecular chain consisting of a series of avidin and biotin-labeled antibodies and enzymes is constructed. The terminal moiety in this complex will be the enzyme lactoperoxidase or horseradish peroxidase, which will serve as a signal generator by catalyzing the radioiodination of the protein substrate bovine serum albumin (BSA). ^{125}I provides a readily measured signal, which is quantitatively proportional to the concentration of the active enzyme and in turn to the concentration of the original NGF antigen in the sample. This novel procedure offers potential for tremendous improvements in both sensitivity and specificity. Additionally, while the assay is being developed for measurement of NGF, the assay scheme being developed will be applicable to virtually any immunologically-recognizable molecule, so that this method can be generalized for measurement of other biologically important substances.

VII-Immune function: Neuroendocrine, behavioral and autonomic correlates:

Populations of recruits or mobilized soldiers are particularly susceptible to infectious diseases that may severely incumber their performance. The incidence of such diseases in these individuals far exceeds their incidence in other populations of grouped individuals. In an attempt to define the cause of such problems, potential correlations among immune system function (troops often receive innoculations at these times), stress responses and other complicating variables must be evaluated. A newly emerging area of biomedical research is directed at this goal, and interactions among immune function, autonomic responses, and the neuroendocrine axis are already known. We recently initiated a series of studies to further evaluate the biological basis of these interactions.

This research has focused on the neuroendocrine modulation of immune function, specifically the primary immune response. In particular, we are investigating the role of prolactin, an anterior pituitary hormone released in a graduated manner in response to stress and possessing known immunomodulatory effects. Prolactin may also be released in response to antigen exposure and be a component of the immune recognition process. The effect of serum prolactin in response to a variety of administered antigens was studied in mice. Additionally, we investigated the effects of inhibition or augmentation of prolactin release at varying timepoints after immunization on the titers of primary IgM antibody elicited. At this time, no definitive results are available. However, it is hoped that this research will shed some light on the mechanisms of stress induced immunosuppresion, and provide a means to more easily identify and quantitate this phenomenon in man.

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A growing body of literature suggests that the endogenous opioid systems may, in addition to their many other effects, play a role in immunomodulation. Prior work in this area has been contradictory, indicating that both opioid agonists and antagonists can inhibit tumor growth. Various investigators have reported that naltrexone, naloxone, and heroin can modify the rate of growth of neuroblastoma or mammary tumor in rodents. To clarify these discrepancies, we examined the effect of chronic treatment with naloxone or morphine on growth of plasmacytoma tumors in mice. This tumor line was chosen since it is not known to be sensitive to opioids (like neuroblastoma) or prolactin (like mammary tumors). Results to date have failed to show a significant effect of morphine or naloxone on the rate of plasmacytoma tumor growth in mice. This may suggest that opiates and their antagonists are not general modulators of immune-system anti-tumor activity, but may interact through other modulatory systems to alter tumor growth.

We also examined the effects of endogenous opioids and their derivatives on the response of leukocytes to chemotaxic peptides. These studies showed that β-endorphin and its non-opioid receptor binding derivative, n-acetyl-β-endorphin, increase chemotactic leukocyte migration at physiological concentrations. Because of the extremely low concentrations required for these effects, these results may be the first to demonstrate a possible role for physiologically released pituitary endorphins.

Future Directions:

Plans for 1985 include the continuing priority of obtaining functional laboratory and office space to afford the opportunity to pursue research objectives outlined in approved and planned research proposals. Studies within the Neuropharmacology Branch will continue within the various subtopics outlined above. Specifically, efforts will be made to define further the biological mediators of circulatory shock, CNS ischemia and their functional interrelationships. At a more fundamental level, mechanisms of receptor interactions will be evaluated, to include possible molecular interactions between opioid receptor systems and adrenergic receptors. The autonomic responses to the neuropeptides cholecystokinin and glucagon will be established, as well as their possible therapeutic effects in experimental models of shock and trauma. The electroencephalographic and convulsant responses to opioid and non-opioid drugs will be extended, with new emphasis on correlations between developmental responses to neurotrophic factors. Blood-brain barrier experiments will continue to elucidate neuroendocrine factors governing access to the brain. From a biochemical perspective, the development of new immunoassay techniques will allow for detection of biological substances such as NGF and other neuromodulators in tissues where concentrations are so low as to have defied standard assay procedures. The influence of NGF on developmental processes, viability of brain

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transplants, and cholinergic function will be extended. Investigations into correlations among immune system function and autonomic responses will be continued, with particular attention given to the possible effects of neuropeptides such as the endogenous opioids and prolactin.

These various biomedical disciplines are all related as interactive components that mediate responses to growth, development and injury. The team of researchers that compose the Neuropharmacology Branch share a common interest in developing a more global picture of biological function, with particular emphasis on defining fundamental physiological processes and their amenability to pharmacological manipulation. Results of these collaborative studies have already provided new understandings of the processes that underly medical disorders as well as new drugs for their treatment. A common thread linking these research areas is their relevance to clinical issues, with particular emphasis on applications within the military medical environment.

Invited Presentations:

John W. Holaday, Ph.D.:

1. First International Meeting of the Italian Society of Endocrinology, Recent Progress in Opioid Research: Central and Peripheral Endorphins, Basic and Clinical Aspects., invited lecturer, Viareggio, Italy, Oct. 1983.

2. First International Meeting of the Italian Society of Endocrinology, Recent Progress in Opioid Research: The Opioid Modulation of the Endocrine Function., invited lecturer, Florence, Italy, Oct. 1983.

3. Twenty-Third Interscience Conference on Antimicrobial Agents and Chemotherapy, invited symposium speaker, 'The role of endorphins in the pathogenesis of septic shock'. Las Vegas, Nevada, October 1983.

4. Sixth Asian-Australian Congress of Neurological Surgery, invited symposium speaker, 'Neuropeptides in experimental spinal cord injury and cerebral ischemia'. Hong Kong, Nov. 1983.

5. Winter Conference on Brain Research, invited symposium participant, 'Psychological and neuroendocrinological influences on immune function', Steamboat Springs, Colorado, Jan. 1984.

6. Winter Conference on Brain Research, invited symposium participant, 'Endogenous opioid systems and autonomic function', Steamboat Springs, Colorado, Jan. 1984.

7. Western Pharmacological Society, symposium chairman, 'Strategies for studying receptor function', Reno, Nevada, Jan. 1984.

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8. Western Pharmacological Society, invited speaker, "Neuropeptides and receptor in experimental shock and trauma. Reno, Nevada, Jan. 1984.

9. Federation of American Societies for Experimental Biology, invited symposium participant, "Mediator Mechanisms in Shock", St. Louis, MO, Apr. 1984.

10. Oral Roberts University, Departmental seminar, Tulsa OK, April 1984.

11. U.S. Army Medical Research and Development Command Workshop on Drugs and Hypovolemic Shock, invited participant, "Naloxone and Thyrotropin Releasing Hormone", San Antonio, TX, March 1984.

12. Medical College of Albany, Dept. Physiology, Distinguished Visiting Scientist, Albany, NY, April 1984.

13. University of Virginia, Dept. of Anesthesiology, Multiple opioid receptors and cardiorespiratory responses, Charlottesville, VA, May 1984.

14. C.I.N.P, Session Chairman - Pharmacological agents in pain therapy; biological and clinical aspects, and Invited symposium speaker - Peptides in the brain; Endogenous opioid systems and autonomic function, Florence, Italy, June 1984.

15. Pathological Society of Great Britain and Irelend, Plenary lecturer, Role of Opioids in endotoxic shock, Leeds, England, June 1984.

16. American Society for Pharmacology and Experimental Therapeutics, Fall Meeting, invited symposium speaker: "Evidence for multiple opiate binding site interactions in vivo", Indianapolis, IN, Aug. 1984.

17. National Navy Medical Center, Intensive Care Dept.: "The use of opioid antagonists in critical care medicine: experimental strategies", Bethesda, MD, Aug. 1984.

18. Walter Reed Army Institute of Research Fellowship Program: "Multiple working hypotheses and strong inference in experimental design", Washington, DC, Aug. 1984.

19. Joint Technology Coordinating Group for Combat Casualty Care, WRAIR: "Rationale for the use of opioid antagonists in septic and hemorrhagic shock", Washington, DC, Sept. 1984.

20. Medical Grand Rounds, Vanderbilt University School of Medicine: "Opiate antagonists and TRH in the treatment of shock and trauma", Nashville, TN, Sept. 1984.

21. Clinical Pharmacology, Vanderbilt University School of Medicine: "Autonomic effects of endogenous opioid systems", Nashville, TN, Sept, 1984.

22. Surgical Research Conference, Vanderbilt University School of Medicine: "Circulatory shock and spinal trauma: Pathophysiological role of endogenous opioid systems", Nashville, TN, Sept, 1984.

Frank C. Tortella, Ph.D.:

1. Lecture at Nicolet Corporation, Lanham, MD., on the applicability of the Nicolet Pathfinder II to clinical and basic EEG research, Madison, WI, Dec. 1983.

2. Invited speaker at the West Pharmacol. Soc. Symposium entitled "Strategies for Studying Receptor Function." Reno, NV, Jan. 1984.

3. Medical pharmacology lecture on Antiepileptics/Analeptics at Temple University School of Medicine, Philadelphia, PA, Feb. 1984.

4. Participant in the WRAIR Psychopharmacology Study Groups. Forest Glen 1984.

William C. Mobley, M.D., Ph.D.:

1. Johns Hopkins University, Neurovirology: "NGF and its Effects on Basic Forebrain Cholinergic Neurons", Baltimore, MD, October 1983.

2. Johns Hopkins University, Neuropathology: "Developmental Studies of NGF Effects on Chorinergic Neurons", Baltimore, MD, January 1984.

3. Massachusetts Institute of Technology - Tub. Sclerosis Assoc.:"Neuronotrophic factors in neurologic disease.", Boston, MA, Spring, 1984.

4. University of Calfornia, San Francisco - Neurology and Pediatrics - "Growth and regeneration of central cholinergic neurons: does NGF have a role?", San Francisco, CA, August 1984.

E. W. Bernton, M.D.:

1. Suburban Hospital, Silver Spring, MD: "Experimental insights into shock therapy", Silver Spring, MD, January 1984.

D. S. Malcolm, Ph.D.:

1. National Naval Medical Center, Intensive Care Conference: "Cholecystokinin: an endogenous opioid antagonist?", Bethesda, MD, August, 1984.

Patents Issued:

1. Holaday, J.W. and Faden, A.I. Narcotic Antagonists in the Therapy of Shock. Patent #4,434,168, Feb.28, 1984.
2. Holaday, J.W. Thyrotropin Releasing Hormone in the Therapy of Shock and as a Central Nervous System Stimulant. Patent #4,426,378, Jan. 17, 1984.

Publications:

1. Belenky, G.L., Gelinas-Sorell, D., Kenner, J.R., and Holaday, J.W. Evidence for delta receptor involvement in the post-ictal antinociceptive responses to electroconvulsive shock in rats. *Life Sci.* 33: 585-586, 1983.
2. Tortella, F.C., Robles, L.E., Holaday, J.W., and Cowan, A. A selective role for d-receptors in the regulation of opioid-induced changes in seizure threshold. *Life Sci.*, 33: 603-606, 1983.
3. Holaday, J.W., and D'Amato, R.J. Multiple opioid receptors: evidence for μu-ϕelta binding site interactions in endotoxic shock. *Life Sci.*, 33: 703-706, 1983.
4. Holaday, J.W., D'Amato, R.J., Ruvio, B.A., Feuerstein, G., and Faden, A.I. Adrenalectomy blocks pressor responses to naloxone in endotoxic shock: evidence for sympatho-medullary involvement. *Circ. Shock*, 11: 201-210, 1983.
5. Holaday, J.W., and Faden, A.I. TRH: Autonomic effects upon cardiorespiratory function in endotoxic shock. *Reg. Peptides*, 7: 111-125, 1983.
6. Holaday, J.W. Opiate antagonists in shock and trauma. *Am. J. Emerg. Med.*, 2: 8-12, 1984.
7. Tortella, F.C., Cowan, A. and Holaday, J.W. Pituitary opioid involvement in electroconvulsive shock-induced postictal electrogenesis and behavioral depression in rats. *Peptides* 5: 115-118, 1984.

8. D'Amato, R.J., and Holaday, J.W. Multiple opiate receptors in endotoxic shock: evidence for delta involvement and mu-delta interactions in vivo. Proc. Natl. Acad Sci. 81: 2898-2901, 1984.

9. Holaday, J.W., and E.W. Bernton, Protirelin (TRH): a potent neuromodulator with therapeutic potential. Arch Int. Medicine, 144: 1138-1140, 1984.

10. Holaday, J.W. Neuropeptides in shock and traumatic injury: sites and mechanisms of action. Neuroendocrine Perspectives, 3: 161-199, 1984.

11. Tortella, F.C., L.E. Robles, J.W.Holaday & A. Cowan, A selective role for δ-receptors in the regulation of opioid-induced changes in seizure threshold. Life Sci. 33, 603-606, 1983.

12. Tortella, F.C., A. Cowan and M.W. Adler. Studies on the excitatory and inhibitory influence of intracerebroventricularly injected opioids on seizure thresholds in rats. Neuropharmacol. 23, 749-754, 1984.

13. Tortella, F.C., & J.W. Holaday. μ and δ opioid receptor interactions in a rat model of drug induced seizures. Proc. West. Pharmacol. Soc. 27, 435-437, 1984.

14. Holaday, J.W., Kenner, J.R., Glatt, C.E. and Long, J.B. Dynorphin: cardiovascular consequences of opioid receptor interactions in normal and endotoxemic rats. Proc. West. Pharmacol. Soc. 27, 429-433, 1984.

15. Holaday J.W. and F.C. Tortella. Multiple opioid recpetors: Possible physiological functions of μ and δ binding sites in vivo. In Central and Peripheral Endorphins: Basic and Clinical Aspects, E.E. Miller and A.R. Genazzani (Eds.), Raven, New York, 1984, pp. 237-250.

16. Holaday, J.W., Gilbeau, P.M., Smith, C.G., and L.L. Pennington. Multiple opioid receptors in the regulation of neuroendocrine responses in the conscious rat and monkey. Opioid Modulation of Endocrine Function, G. Delitala et al (Eds.), Raven Press, NY, 1984, pp. 21-32.

17. Kitt, C.A., Mobley, W.C., Struble, R.G., Cork, L.C., Hedreen, J.C., Wainer, B.H., and Price, D.L., 1984. Evidence for cholinergic processes in neuritic plaques of aged primates. Neurology 34 (Suppl. 1): 121-122.

18. Kitt, C.A., Mobley, W.C., Struble, R.G., Walker, L.C., Cork, L.C., Becher, M.W., Joh, T. and Price, D.L. 1984. The contribution of catecholaminergic systems to neurites in the plaques of aged primates. Ann. Neurol. 16, 118.

19. Holaday, J.W. Internal and external opioids update. U.S. Journal of Drug and Alcohol Dependence, 8,19, 1984.

20. Holaday, J.W. Endogenous opioid systems and autonomic function. Int. Cong. Neuropsychopharmacology G. Racagni et al (Eds) Raven Press, 1984.

21. Long, J.B., Youngblood, W.W., and Kizer, J.S. Effects of castration and adrenalectomy on in vivo rates of tryptophan hydroxylation and levels of serotonin in microdissected brain nuclei of adult male rats. Brain Res. 277, 289-297, 1983.

Manuscripts submitted or in press:

1. Holaday, J. W. Endorphins in: Current Concepts, in press.

2. Holaday, J.W., Black, L.B., and Long, J.B. Neuropeptides in shock and trauma. Clinics in Critical Care Medicine, Chernow, B., ed., in press.

3. Belenky, G.L., Tortella, F.C., Hitzemann, R.J. and Holaday, J.W. The role of endorphin systems in the effects of single and repeated electroconvulsive shock. In: ECS: Basic Mechanisms. R.M. Belmaker, B. Lerer, and R.D. Weiner eds., John Libbey and Co., London, in press, 1983.

4. Tortella, F.C., Robles, L, Holaday, J.W., and Cowan, A. ICI 154,129, a putative δ opiate receptor antagonist, raises seizure threshold in rats. Eur. J. Pharmacol., in press.

5. Bernton, E.W., Long, J.B., and Holaday, J.W. Opioids and neuropeptides: mechanisms in circulatory shock. Fed. Proc. in press, 1984.

6. Chernow, B. and Holaday, J.W. The pathogenisis of septic shock, J.A.M.A. in press, 1984.

7. Gilbeau, P.M., Almirez, R.G., Holaday, J.W,. and Smith CG. The role of endogenous opioids peptides in the control of androgen levels in the male non-human primate J. Andrology, in press, 1984.

8. Sampson, J.A., Bass, B.L., Harmon, J.W., and Holaday, J.W. Naloxone reduces renal blood flow in rabbit hemorrhagic shock. Surgical Forum, in press, 1984.

9. Tortella, F.C., Long J.B., and Holaday, J.W., Endogenous opioid systems: Physiological role in the self-limitation of seizures. Brain Res. submitted, 1984.

10. Holaday, J.W., Pennington, L.L., and Ward S.J. Mu and delta opioid receptors and endocrine responses: mu receptors mediate prolactin release. *Peptides*, submitted, 1984.

11. Gilbeau, P.M., Almirez, R.G., Holaday, J.W,. and Smith C.G. Opioid effects on blood concentrations of luteinizing hormone and prolactin in the adult male rhesus monkey *J. Clin. Endocrinol. and Metab.*, (in press).

12. Tortella, F.C., L. Robles, H.I. Mosberg & J.W. Holaday, Electroencephalographic assessment of the role of δ receptors in opioid peptide-induced seizures, *Neuropeptides* (in press).

13. J.W. Holaday, G.L. Belenky, J.R. Kenner, F.C. Tortella, B. Hitzemann, S. Blatt & R.J Hitzemann, Repeated electrocenxulsive shock and morphine tolerance: Sodium effects upon increased opioid binding sites in rat brain membranes (submitted).

14. Price, D.L., Kitt, C.A., Hedreen, J.C., Whitehouse, P.J., Struble, R.G., Cork, L.C., Walker, L.C., Mobley, W.C., Salvaterra, P.M. and Wainer, B.H. Basal forebrain cholinergic systems in primate brain: anatomical organization and role in the pathology of aging and dementia. In: *Dynamics of Cholinergic Function*, I. Hanin (Ed), New York, Plenum Press (in press).

15. Kitt, C.A., Price, D.L., Struble, R.G., Cork, L.C., Wainer, B.H. and Mobley, W.C., Direct evidence for cholinergic neurites in neocortical plaques. *Science* (in press).

16. Kitt, C.A., Cork, L.C., Mobley, W.C., Struble, R.G., Walker, L.C., Joh, T.H. and Price, D.L. Catecholaminergic neurites and senile plaques in prefrontal cortex of aged nonhuman primates (submitted).

17. Sapun-Malcolm, D., Farah, J.M. Jr., Mueller, G.P., Serotonin and dopamine independently regulate pituitary beta-endorphin release *in vivo* (submitted).

18. Mueller, G.P., Pettibone, D.J., Farah, J.M.Jr., Sapun-Malcolm, D., *In vivo* release of beta-endorphin immunoreactivity by rat pars distalis: inhibition by glucocorticoids (submitted).

19. Farah, J.M. Jr., Sapun-Malcolm, D., Mueller, G.P., Evidence for dopaminergic inhibition of pars distalis and pars intermedia secretions of pituitary beta-endorphin-like immunoreactivity (submitted).

20. Farah, J.M. Jr., Sapun-Malcolm, D., Mueller, G.P., Regulation of anterior pituitary release of immunoreactive beta-endorphin *in vivo* by dopamine receptor subtypes (submitted).

21. Mobley, W.C., 1984. The Guillain-Barre Syndrome Study Group (1984). Plasmapheresis & acute GBS (submitted).

22. Long, J.B. and Holaday, J.W., Blood-brain barrier: endogenous modulation by adrenal cortical function. Science (submitted).

23. Long, J.B., Ruvio, B.A., Glatt, C.E., and Holaday, J.W., ICI 174864, a putative δ opioid antagonist, reverses endotoxemic hypotension: pretreatment with dynorphin 1-13, a κ agonist, blocks this action. Neuropeptides (in press).

24. Holaday, J.W. Opioid antagonists in septic shock. Septic shock M.A. Sande and R.K. Root (eds) Churchill Livingstone Inc., New York, NY (in press).

25. Tapp, W.N., Holaday, J.W. and Natelson, B.H. Ultradian glucocorticoid rhythms in monkeys and rats continue during stress. Am. J. Physiol. (in press).

26. Malcolm, D.S. and Holaday, J.W. Opioid peptides and their antagonists: A role in respiratory function. Seminars in Respiratory Medicine (in press).

27. Long, J.B., Youngblood, W.W., and Kizer, J.S. Possible involvement of serotonergic neurotransmission in neurotensin but not morphine analgesia. Brain Res. (in press).

Abstracts:

1. Ruvio, BA, Schneider, H, Bernton, E. and Holaday, JW Failure of naloxone to reverse the hypotensive effects of cobra venom factor in rats. Circ. Shock 13, 46 (1984).

2. Bernton, E.W., Black, L.E. and Holaday, J.W. Effects of morphine tolerance on endotoxic shock hypotension in rats and its reversal by naloxone. Fed. Proc. 43, 1026, 1984.

3. Holaday, J.W., Kenner, J.R. and Glatt, C.E. Dynorphin: cardiovascular effects and opioid receptor interactions in normal and endotoxemic rats. Fed. Proc. 43, 653, 1984.

4. Tortella, F.C., Long, J.B., Robles, L. and Holaday, J.W. Progressive decrease in sezure severity produced with intermittent electroconvulsive shock: Involvement of endogenous opioid systems. Soc. Neurosci. Abstr. 9, 289, 1984.

AVAILABLE

5. Long, J.B., Lake, C.R., Reid, A., Ruvio, B.A. and Holaday, J.W. Effects of naloxone and TRH on plasma catecholamines and arterial pressure in normal and endotoxemic rats. Soc. Neurosci. Abstr. 9, 107, 1984.

6. Holaday, J.W., Pennington, L. and Ward, S.J. Selective μ and δ receptor antagonists and neuroendocrine responses to morphine: Evidence for μ receptors in prolactin release. Soc. Neurosci. Abstr. 9, 744, 1984.

7. Holaday, J.W., Kenner, J.R. and Glatt, C.E., Dynorphin: cardiovascular consequence of opioid receptor interactions in normal and endotoxemic rats. West. Pharm. Soc., 1984.

8. Bernton, E.W., Black, L.E., Ruvio, B.A. and Holaday, J.W. Morphine tolerance enhances endotoxic shock susceptability and exaggerates the pressor response to naloxone in conscious rats. Circ. Shock 13, 45, 1984.

9. Chernow, B., Reed, L., Geelhoed, G., Meyerhoff, J., Lake, C.R., Beardsley, D. and Holaday, J.W. Verapamil antagonizes glucagon's chronotropic effect. Circ. Shock 13, 96, 1984.

10. Mobley, W.C., Kitt, C.A., Struble, R.G. and Price, D.L. Displacement of myelin sheaths by the neuritic plaques of Alzheimer's disease. Soc. Neurosci. Abstr. 9, 270, 1984.

11. Mobley, W.C. 1984. Neuronotrophic factors in neurologic disease. Abstr., Tuberous Sclerosis Res. Workshop, 39-40.

12. Long, J.B., Ruvio, B.A., and Holaday, J.W., ICI 174864, a novel δ antagonist reverses endotoxic shock: pretreatment with dynorphin 1-13, a κ agonist, blocks this therapeutic effect, International Narcotics Research Conference, Cambridge, U.K., 1984.

13. Long, J.B., Holaday, J.W., and Tortella, F.C., Effects of Adrenalectomy and Hypophysectomy on ECS-induced postictal seizure protection. IUPHAR 9th International Congress of Pharmacology (London, UK), 1460P, 1984.

14. Holaday, J.W. and Tortella, F.C., In Vivo evidence for functional interactions among κ, μ and δ opioid binding sites. Int. Cong. Neuropsychopharm., 1984.

15. Tortella, F.C. and Holaday, J.W., μ and δ opioid receptor interactions in a rat model of experimental seizures. West Pharmacol. Soc., 1984.

16. Tortella, F.C., Long, J.B., Robles, L. and Holaday, J.W., Physiological tolerance to endogenous opioid activation: Inhibition of seizure protection in morphine-tolerant rats. Fed. Proc. 43, 936, 1984.

17. Tortella, F.C., Robles, L., Mosberg, H.I. and Holaday, J.W., Electroencephalographic assessment of the role of δ receptors in opioid peptide-induced seizures. International Narcotics Research Conference, (Cambridge UK), p. 19, 1984.

18. Holaday, J.W., Belenky, G.L., Kenner, J.L., Tortella, F.C., Hitzeman, B., Blatt, S, and Hitzemann, R.J., Repeated electroconvulsive shock and morphine tolerance: Sodium effects upon increased opioid binding sites in rat brain membranes. International Narcotic Research Conference (Cambridge, UK), p. 8, 1984.

19. Chernow, B., Clapper, M., Malcolm, D., Holaday, J., Glucagon has a chronotropic effect in rats. Am. Fed. Clin. Res., 1984.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 304906
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DRA&E(AR) 636
3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING:
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62777A	3E162777A879	AB	048 WWJQ
b. CONTRIBUTING				
c. CONTRIBUTING/STOG-82/83-6.2/2				

11. TITLE (Precede with Security Classification Code)
(U) Biobehavioral Foundations of Continuous Military Performance

12. SUBJECT AREAS
0510 Psychology 0616 Physiology 0619 Stress Physiology

13. START DATE: 83 10
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING ORGANIZATION: DA | | C
16. PERFORMANCE METHOD: In-House

17. CONTRACT GRANT
a. DATE EFFECTIVE:
EXPIRATION:
b. CONTRACT GRANT NUMBER:
c. TYPE:
d. AMOUNT:
e. KIND OF AWARD:
f. CUM/TOTAL:

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	1.0	41
85	1.0	65

19. RESPONSIBLE DOD ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
b. ADDRESS (include zip code): Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL: Top, F H Jr
d. TELEPHONE NUMBER (include area code): (202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME: Walter Reed Army Institute of Research
Division of Neuropsychiatry
b. ADDRESS: Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR: Leu, J R
d. TELEPHONE NUMBER (include area code): (202)-576-3003
f. NAME OF ASSOCIATE INVESTIGATOR (if available): Elsmore, T F
g. NAME OF ASSOCIATE INVESTIGATOR (if available): Kant, C J

21. GENERAL USE
F I N A
MILITARY/CIVILIAN APPLICATION H

22. KEYWORDS (Precede EACH with Security Classification Code) (U)RAM III;(U)Physiology;(U)Performance:(U)Lab Animals (U)Rats;(U)Neurophysiology;(U)Neuropsychiatry;(U)Chronobiology;(U) Continuous Operations:

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Investigations in this work unit will address biological and behavioral factors underlying neuropsychiatric problems encountered under conditions of sleep deprivation, unusual or extended work schedules, and prolonged environmental or emotional stress. Methods of counteracting performance decrements will also be investigated. There is military relevance in this research.

24. (U) Animal models of military performance will be developed using the techniques of operant and respondent conditioning, behavioral pharmacology, and neurophysiology. Various behavioral measures will be assessed during continuous performance testing using computer-controlled environments. Changes in physiological indices of stress will be routinely examined during the continuous performance testing cycle. Changes in quantitative and qualitative measures of performance will be determined during a variety of performance conditions, including graded stressors, disruption of circadian rythyms, and sleep loss. Models will be developed or chosen which permit extrapolation from animal to human performance with a minimum of difficulty. Effectiveness of various measures to counteract the effects of continued stress will be evaluated. Behavioral, pharmacological, chronobiological, and dietary treatments will be considered.

25. (U) 83 10 - 84 09 Major Findings: A laboratory for studying the effects of continuous stress has been established. Computer programs and experimental procedures have been developed. Initial studies have shown that rats exposed to 72 hours of moderate stress are not serverly impaired in performance on several standard behavioral tasks. More complex tasks appear to be more sensitive, being affected more quickly and more strongly than simple tasks. These studies have provided a basis for a working model of continuous performance in rats. Conducted a 2-day workshop on "Pharmacological Optimization of Performance", which included internationally recognized scientific authorities in the areas of Memory and Cognitive Functioning, Sleep and Circadian Rythyms, Fear and Anxiety, and Strength and Fatigue. For technical report see Walter Reed Army Institute of Research Annual Report, 1 Oct 83 - 30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

Project: 3E162777A879 MEDICAL FACTORS ENHANCING SOLDIER EFFECTIVENESS

Work Unit 48: Biobehavioral Foundations of Continuous Military Performance

Investigators:
Principal: Leu, CPT, J.R.
Associate: Elsmore, T.F., Ph.D.; Kant, G.J., Ph.D.

Objectives:

Investigations in this work unit are designed to address the biological and behavioral factors leading to reduced performance during continuous military operations. Serious reductions in performance are common under conditions of sleep deprivation, unusual or extended work schedules, and prolonged environmental or emotional stress. Methods for counteracting these performance decrements will also be investigated. A primary goal is the development of animal testing procedures which model the continuous activity and stresses inherent in military operations. Work on this project uses the knowledge and techniques of psychology, neuropharmacology, chronobiology, and neurophysiology.

Progress:

In this first year for the work unit a laboratory was established for studying the effects of continuous stress in small rodents. Equipment and supplies were procured which allow simultaneous monitoring of six animals using computer-controlled testing chambers. Computer programs and experimental procedures have been developed for a chronic avoidance task that requires animals to pull a ceiling chain when either of two successive warning signals is presented in order to prevent the onset of electric footshock. The initial shock is barely detectable with the level increasing incrementally over several seconds before terminating at a preset maximum intensity. Pulling the ceiling chain during shock presentation will immediately terminate the shock. The frequency of avoidance trials and the duration of sessions can be easily and systematically varied. Initial studies used 72 hour periods of exposure to the chronic avoidance task.

Quantitative and qualitative measures of performance were examined during several challenge conditions. Included were gradations in the frequency and intensity of avoidance trials which resulted in disruptions of circadian rythyms and loss of sleep. Initial studies show that rats exposed to 72 hours of moderate stress are not serverly impaired in performance on several standard behavioral tasks. Complex tasks appear to be more sensitive, being affected more quickly and more strongly than simple tasks. These studies provide a basis for a working model of continuous performance in rats.

A 2-day workshop on "Pharmacological Optimization of Performance" was conducted. This workshop included internationally recognized scientific authorities in the areas of Memory and Cognitive Functioning, Sleep and Circadian Rythyms, Fear and Anxiety, and Strength and Fatigue. This workshop provided valuable information and insights into a number of areas currently being investigated. The direction of research in this area under several projects has already been influenced by the information obtained at this workshop and it may prove to be one of the most significant such gatherings we have sponsored in recent years. Conclusions and recommendations from this workshop will be published.

Future objectives:

Additional equipment will be procured to enlarge the capacity to eight animals. Systematic testing will continue to determine the most appropriate parameters for the chronic avoidance model of continuous performance. Additional behavioral tasks will be evaluated to determine which are most sensitive, reliable, and meaningful for this model. The goal is a model which permits extrapolation from animal to human performance with a minimum of difficulty. The effectiveness of various measures to counteract the stress associated with chronic avoidance task will be evaluated. Behavioral, pharmacological, chronobiological, and dietary treatments will be considered. Neurochemical and hormonal indices of stress will be measured in animals at various times during and following periods of chronic avoidance. Changes in these measures will be correlated with changes in performance.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA305991
2. DATE OF SUMMARY: 84 10 01
REPORT CONTROL SYMBOL: DD-DR&E(AR) 636

3. DATE PREV SUM'RY: 83 10 01
4. KIND OF SUMMARY: D. Change
5. SUMMARY SCTY: U
6. WORK SECURITY:
7. REGRADING: U
8. DISB'N INSTR'N: CX
9. LEVEL OF SUM: A WORK UNIT WRAIR

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62777A	3E162777A879	AA	049 WWQJ
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 52/83-6.2/2			

11. TITLE (Precede with Security Classification Code)
(U) Medical Factors Limiting Rapid Deployment

12. SUBJECT AREAS
0619 Stress Physiology 0510 Psychology

13. START DATE: 83 10
14. ESTIMATED COMPLETION DATE: Cont
15. FUNDING ORGANIZATION: DA
16. PERFORMANCE METHOD: C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	1.0	50
85	2.0	40

19. RESPONSIBLE DOD ORGANIZATION
a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, D.C 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR
d. TELEPHONE NUMBER (include area code) 202-576-3551

20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research
US Army Medical Research Unit-Ft. Bragg
b. ADDRESS Box 338, USA MEDDAC
Ft. Bragg, N.C. 28307-5000
c. NAME OF PRINCIPAL INVESTIGATOR Harris, J
d. TELEPHONE NUMBER (include area code) (919) 396-9432
f. NAME OF ASSOCIATE INVESTIGATOR (if available) Holgate, S
g. NAME OF ASSOCIATE INVESTIGATOR (if available) Russell, C

21. GENERAL USE FINA
MILITARY/CIVILIAN APPLICATION: H

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Stress ; (U) Deployment ; (U) Readiness ; (U) RAM I (U) Volunteers

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) Examine current health problems identified by XVIII Airborne Corps units or WRAIR scientists as impeding deployment readiness, with special emphasis on behavioral and psychological consequences of stress. This research is of military importance.

24. (U) The method of epidemiology, including records analysis, population and demographic analysis, questionnaires, field and cohort studies, and various observational methods, are employed to develop requisite data.

25. (U) 83 10 - 84 09 Data was collected from soldiers of the 82nd Airborne and Ranger Battalions who participated in Operation Urgent Fury (Grenada). Interviews were content analyzed over several iterations and reported as lessons learned regarding psychological, behavioral, and medical issues pertaining to the rapid deployment of combat troops. Psychosocial questionnaire data continued to be collected, analyzed and reported on 82nd Airborne and 101st Airborne (Air Assault) Battalions deployed to the Sinai as members of the MFO contingent. Participant observer data on interpersonal relations in infantry rifle squads were collected by a two member research team which deployed with an 82nd Airborne Battalion to Gallant Eagle 84. Data related to family violence were collected on Fort Bragg units. A set of demographic statistics of perpetrators and victims, types of services utilized, and stressors preceding the incident were reported. Data collection will continue in the area of family violence. New efforts to study "bonding" processess of combat, combat support and combat service units will begin. An epidemiological study of overweight soldiers will be conducted at the request of the XVIII Abn Corps. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

PROJECT 3E162777A879 MEDICAL FACTORS ENHANCING SOLDIER EFFECTIVENESS

WORK UNIT: 049 Medical Factors Limiting Rapid Deployment

Investigators:

Principal: Jesse J. Harris, COL, MSC

Associates: Stanley H. Holgate, MAJ
Christine M. Russell, Ph. D.

1. Problem and Objectives

This unit examines the dynamics of those specific factors within military organizations and environments that conduce to psychiatric illness, operate to produce psychiatric casualties and lead to the generation of dysfunctional behaviors and decrements in military performance. These studies have direct relevance for the development of programs of intervention and prevention and the development of effective techniques for the minimization of psychiatric casualties.

2. Progress

Interviews were conducted with 139 82nd Airborne personnel and 62 Rangers who deployed to Grenada as part of Operation Urgent Fury. The interviews were designed to obtain insights into combat stressors present during Operation Urgent Fury. The interviews were content analyzed over several iterations for psychosocial themes related to stress. The findings were developed as lessons learned to be used by commanders in reducing or preventing similar stress in future combat operations. At the request of the Chief of Staff, XVIII Airborne Corps, the Commander, USAMRU-FB deployed to Grenada for approximately two weeks in November 1983.

An officer and an enlisted service member deployed with an airborne infantry maneuver unit to California for Exercise Gallant Eagle 1984. They deployed as participant observers for purposes of observing interpersonal behaviors within selected squads. The observations have been transcribed and will be content analyzed for identifying any stressors which could impede troop readiness for deployment. In addition a questionnaire designed to tap variables associated with interpersonal relations within the company was administered to service members in grade E5 and below. The data were grouped by veterans and non-veterans of the Grenada deployment. The veterans' response patterns reflected greater cohesion and a stronger buddy system than the non-veterans' responses. The differences may have been artifacts of the veterans somewhat higher grade and longer time in service, however. Thus, more data from matched groups of veterans and non-veterans are needed.

A civilian sociologist assigned to the Department of Military Psychiatry, WRAIR and attached to USAMRU-FB has begun preliminary research on Family Violence. Thus far the study has isolated a set of demographic statistics of perpetrators and victims, types of services utilized and stressors preceding the incident.

3. Future Work

A protocol is being prepared to systematically study the role of interpersonal relations in deployment readiness in combat, combat support, and combat service support units. The study is being designed to examine both horizontal and vertical bonding to include a look at cross-gender bonding under different levels of stress.

4. Publications

Segal, D., Jesse J. Harris, Joseph M. Rothberg, David H. Marlowe, "Paratroopers As Peace Keepers" in ARMED FORCES AND SOCIETY, Summer 1984, pp 487-506.

Harris, Jesse and David Segal, "Observation from the Sinai: The Boredom Factor" in ARMED FORCES AND SOCIETY, Fall 1984, (In Press).

Rothberg, Joseph, Jesse Harris, Richard Pickle and Linda Jellen, "Stress of Transitions: Illness Reports and the Health of the United States Battalions During the Initial Sinai MFO Deployment."

5. Presentations

Harris, Jesse J., "The Grenada Experience" presented to "Stress Research and Training Meeting" - Ft Benjamin Harrison, Indiana, 3 April 1984.

"Experiences in the Sinai" presented to Inter-University Seminar of the Armed Forces, Washington, D.C. , 26-27 April 1984.

"Health Problems in the Sinai: A Social Workers' View" at the National Association of Social Workers' Health Conference - Washington, D.C., 11 June 1984.

"Issues of Stress in the Sinai" presented to the Combat Stress Workshop at Ft Sam Houston, Texas, 19 September 1984.

PROJECT 3M463751D993

MEDICAL DEFENSE AGAINST CHEMICAL WARFARE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OH 0610	2. DATE OF SUMMARY 84 10 01	*REPORT CONTROL SYMBOL* *DD-DR&E(AR) 636*

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63751A	3M463751D993	AG	061 WWM3
b. ~~CONTRIBUTING~~	63751A	3M463751D993		
c. ~~CONTRIBUTING~~	CARDS			

11. TITLE *(Precede with Security Classification Code)*
(U) Clinical and Ancillary Studies for Antiradiation Drug Development

12. SUBJECT AREAS
0605 Clinical Medicine 0615 Pharmacology

13. START DATE 81 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	2.0	254
85	2.0	292

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)* Washington, DC 20307-5100	b. ADDRESS Division of Experimental Therapeutics Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR HEIFFER, M H
d. TELEPHONE NUMBER *(include area code)* (202)-576-3551	d. TELEPHONE NUMBER *(include area code)* (301)-427-5393
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* FLECKENSTEIN, L g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* SCHUSTER, B G

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Pharmacology; (U) Antidotes; (U) Toxicity; (U) Pharmacokinetics; (U) Quantitation Methodology; (U) RAD IV; (U) Volunteers; (U) Dogs

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) The goal of this work is to develop clinical information on candidate antiradiation drugs that have progressed successfully through preclinical studies. This information is directed toward further development of these drugs through clinical investigations to demonstrate their efficacy and limitations in human subjects using models where necessary. These studies support development of a radioprotective drug for use on the nuclear battlefield.

24. (U) The first questions addressed with these studies concern determinations of doses of effectiveness and minimal toxicity in man. This work relies on animal model studies since humans cannot be intentionally irradiated. Pharmacokinetic investigations in animal models are conducted using sensitive and specific analytical chemical methods. The resulting information is then applied to similar clinical studies in human subjects. Finally, experiments are conducted to determine the optimal dosage forms and route of administration of these drugs for their intended purpose.

25. (U) 83 10 - 84 09 The focus of this work continues to be on producing a pharmacokinetic profile and dosage form of WR 2721 for use in man. Pharmacokinetic studies have been conducted in the beagle dog and rhesus monkey in support of this work. Utilizing the high pressure liquid chromatography methods for assay of WR 2721 and WR 1065, preliminary pharmacokinetics and bioavailability of candidate microencapsulated formulations of WR 2721 have been determined. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE.

Project 3M463751D993 MEDICAL DEFENSE AGAINST CHEMICAL WARFARE

Work Unit 061 Clinical and Ancillary Studies for Antiradiation Drug Development

Investigators:
Principal: Melvin H. Heiffer, Ph.D.
Associate: LTC B.G. Schuster, MD, L. Fleckenstein, Pharm.D.

1. Description.

The goal of this work is to develop clinical information on candidate antiradiation drugs that have progressed successfully through preclinical studies. This information is directed toward further development of these drugs through clinical investigations to demonstrate their efficacy and limitations in human subjects, using animal models where appropriate. These studies support the development of radioprotective drugs for use by our troops on the nuclear battlefield.

2. Progress.

The principal effort of this work has been and continues to be on the pharmacokinetic profiling and dose improvement of WR 2721. Pharmacokinetic studies have been conducted in the beagle dog and rhesus monkey utilizing the microencapsulated dosage form of WR 2721 and the recently developed high pressure liquid chromatographic analysis for WR 2721. Following i.v. administration of radioprotectant doses of WR 2721 (150 mg/kg) to the beagle dog, the drug profile in the plasma was best described with an open two-compartment model. In four experiments, the terminal half-life ranged between 10 and 21 minutes. The volume of distribution is small (about 15% of body weight), and the drug is rapidly cleared from the body (10-14 ml/min/kg). Comparative studies are underway in the rhesus monkey.

Initial tests of WR 168,643, an analog of WR 2721 with a trisulfide linkage, has demonstrated transient tachycardia, tachypnea, hypotension, myocardial contractility depression and respiratory alkalosis when administered i.v. to dogs. It was interesting to note that tissue hypoxia was not seen; this has been postulated by some to be one mechanism of action of the phosphorothioate radioprotectors.

3. Future Work.

Pharmacokinetic profiling of i.v. and orally administered WR 2721 in the rhesus monkey will continue. The resultant data will be compared to that obtained from the beagle to determine if the microencapsulated form of WR 2721 is likely to produce efficacious blood levels of the drug for radioprotective purposes.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION DA OH 0609	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636

3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX	9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63751A	3M463751D993	AE	062 WWM3
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CA			

11. TITLE (Precede with Security Classification Code)
(U) Clinical and Ancillary Studies for Anti-Chemical Warfare Drug Development

12. SUBJECT AREAS
0605 Clinical Medicine 0615 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	CONT	DA	C. In-House

17. CONTRACT GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	3.0	206
85	3.0	292

19. RESPONSIBLE DOD ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS (include zip code)
Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL
TOP, F H JR
d. TELEPHONE NUMBER (include area code)
(202)-576-3551

20. PERFORMING ORGANIZATION
a. NAME
Walter Reed Army Institute of Research
b. ADDRESS
Division of Experimental Therapeutics
Washington, DC 20307-5100
c. NAME OF PRINCIPAL INVESTIGATOR
HEIFFER, M H
d. TELEPHONE NUMBER (include area code)
(301)-427-5393
f. NAME OF ASSOCIATE INVESTIGATOR (if available)
PAMPLIN, C L
g. NAME OF ASSOCIATE INVESTIGATOR (if available)
LOWENSOHN, H S

21. GENERAL USE
FINA
MILITARY/CIVILIAN APPLICATION
H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Pharmacology; (U) Antidotes; (U) Toxicity; (U) Pharmacokinetics; (U) Quantitation Methodology; (U) RAM V

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The purpose of this research is to develop clinical information on candidate chemical warfare agent antidotes that have progressed successfully through preclinical studies. This information is directed toward further development of these antidotes through clinical investigations to demonstrate their efficacy and limitations in human subjects. This research is directed toward ultimate fielding of protective and treatment drugs for use on the chemical battlefield.

24. (U) The first questions addressed with these studies concern determinations of toxic and effective doses in man. This work is supported by prior studies in animal models. Pharmacokinetic investigations in animal models are conducted using sensitive and specific analytical chemical methods. The resulting information is then applied to similar clinical studies in human subjects. Finally, experiments are conducted to determine the optimal dosage forms and route of administration for these drugs for their intended purposes.

25. (U) 83 10 - 84 09 An accelerated stability study of 2-PAM chloride and atropine in an autoinjector has begun. The study will provide a useful estimate of the shelf-life at room temperature for this drug combination. The degradation products of 2-PAM chloride in cartridges stored for 8-10 years at room temperature have been determined with decomposition products identified by comparison of UV spectra. A manuscript reviewing the cardiovascular effects of atropine has been submitted for publication. A protocol testing the effect of atropine on cardiovascular efficacy of conscious dogs has begun. A Phase I pharmacokinetic study in human volunteers given four oral doses of pyridostigmine nears completion. Preliminary evaluation of the data suggests large inter-individual variation in red blood cell cholinesterase inhibition by pyridostigmine. A Phase I multiple dose study of pyridostigmine to determine inter-individual variation at steady-state has been written and will be completed in the next year. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 1498, 83 MAR
EDITION OF MAR 68 IS OBSOLETE

Project 3M463751D993 MEDICAL DEFENSE AGAINST CHEMICAL WARFARE

Work Unit 062 Clinical and Ancillary Studies for Anti-Chemical Warfare Drug Development

Investigators:
Principal: Melvin H. Heiffer, Ph.D.
Associate: LTC C. Pamplin, MC, H.S. Lowensohn, Ph.D., G.J. McCormick, Ph.D.
Assistant: J. Notsch

1. Description.

The goal of this work is to obtain the necessary information to support the granting of a Notice of Claimed Investigational Exemption for a New Drug (IND) by the Food and Drug Administration (FDA) for each candidate chemical warfare antidote or protectant. The development of such drugs requires the efforts of a highly integrated multidisciplinary team to undertake a broad range of preclinical and clinical pharmacological studies.

2. Progress.

A new accelerated stability study of 2-PAM-Cl in autoinjector cartridges, alone or in the presence of atropine, is presently in progress to determine the useful shelf-life of these cartridges. Analytical procedures have been developed to measure these drugs and their decomposition products. These methods employ high pressure liquid chromatography (HPLC) to measure 2-PAM-Cl and gas chromatography to measure atropine. In related studies, three decomposition products of 2-PAM-Cl have been discovered in 8-10 year old cartridges. Methods have been developed to quantitate two of these by HPLC and work is in progress on the third. Tentative identifications have been made based on UV spectral comparisons with standards.

In other work, preliminary studies have been undertaken to examine the cardiovascular effects of atropine on conscious canines trained to exercise on a treadmill. Initial baseline results on resting animals indicate that the peak heart rate response rapidly develops tachyphylaxis to repeated injections of atropine. These results also indicate a considerable amount of variation in this response among individual subjects.

3. Future Work.

The accelerated stability studies of 2-PAM-Cl with and without atropine continue with the goal of finding a compatible mixture of these two drugs that can be fielded in a single autoinjector. Work on the cardiovascular effects of atropine in the canine model has been suspended as the result of DA direction.

4. Publications.

Lowensohn, H.S. Atropine's effects upon the muscarine controls: The heart and its systemic output. Submitted to Physiological Reviews.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION DA OC 6478	2. DATE OF SUMMARY 841001	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
3. DATE PREV SUM'RY 831001	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX
					9. LEVEL OF SUM A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63751A	3M463751D993	AB	063 WWM8
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CARDS			

11. TITLE (Precede with Security Classification Code)
(U) Development of Antiradiation Drugs

12. SUBJECT AREAS
0703 Organic Chemistry 0615 Pharmacology 0603 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
7810	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	2.0	206
85	2.0	347

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Experimental Therapeutics
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H Jr	c. NAME OF PRINCIPAL INVESTIGATOR DAVIDSON, D E
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (301)-427-5411
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U)Drug Development; (U)Antiradiation Drugs; Radiation Protection; (U)Ionizing Radiation; (U)Chemical Synthesis; (U)RAM ✓

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To develop new drugs with protective activity against injury to military personnel in the event of exposure to ionizing radiation.

24. (U) Potentially active drugs will be identified and obtained by synthesis or purchase. Candidate drugs will be tested in laboratory model systems to establish protective efficacy, mechanisms of pharmacological effects, effects on physiological responses and pharmacokinetic characteristics. Information is used in guiding new drug synthesis and selecting candidate drugs for clinical trials.

25. (U) 8310-8409 Fifty-six compounds, including phosphorothioates (analogs of WR 2721), amidinium compounds, bis-sulfinates, thiazolidines and dithioacids, have been synthesized and submitted as candidate radioprotectant compounds. The extramural synthesis program is conducted under five contracts and testing is done in an extramural program consisting of seven contracts which allow evaluation of compounds in vivo and in vitro. In the primary test screen for survival of mice receiving lethal exposure to radiation, 55 compounds were studied by intraperitoneal administration. Of these, four compounds gave evidence of efficacy (90-100 percent protection). In further studies by oral administration, one compound, WR 249914, an amidinium, gave evidence of protection (80 percent) at 600 mg/kg. Test procedures under development include a screening technique dependent upon the enzymes glutathione peroxidase and glutathione-5-peroxidase. These enzymes have been isolated in gram quantities and are in process of purification. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83-30 Sep 84.

AVAILABLE

PROJECT: 3M463751D993 MEDICAL DEFENSE AGAINST CHEMICAL WARFARE

WORK UNIT: 063 Development of Antiradiation Drugs

INVESTIGATORS:

Principal: COL David E. Davidson, Jr., VC

Associate: LTC William H. Amos, Jr., MS
Dr. Melvin H. Heiffer
Dr. Gerald J. McCormick
Dr. David L. Klayman
Dr. Lawrence L. Fleckerstein
Dr. Hikmat A. Musallam
Mr. William Y. Ellis

PROBLEM AND OBJECTIVES:

There are no antidotes to radiation exposure which are available to protect U.S. military personnel in the event of nuclear warfare. The objective of this program is the development of a radioprotective drug which will provide partial protection against both prompt and fallout radiation exposure. WR 2721, a phosphorothioate compound, is a promising candidate but requires developmental effort to improve its oral effectiveness, to minimize undesirable side effects, and to increase the duration of its protective effect. In addition to these developmental studies with WR 2721, identification and development of other compounds with better characteristics will be pursued.

PROGRESS:

The radioprotector program is conducted by extramural contracts with twelve laboratories, collaborative studies with other U.S. and NATO government institutions and by limited intramural research.

The extramural synthesis program is conducted under five contracts. Fifty-six candidate radioprotective compounds have been synthesized for testing, including phosphorothioates (analogs of WR 2721), amidinium compounds, bis-sulfinates, thiazolidines, and dithioacids, and representative compounds of other chemical classes. As an adjunct to the extramural program, a new method was developed by intramural research for the synthesis of tertiary amines for application in the extramural preparation of certain types of antiradiation compounds.

AVAILABLE

Biological efficacy testing of candidate compounds is conducted in an extramural program consisting of seven contracts which allow study of compounds in both _in vivo_ and _in vitro_ test systems. In the primary test screen, efficacy is assessed as the ability to protect mice against exposure to lethal levels of gamma radiation (1000 rads) and is measured as survival achieved after intraperitoneal administration of the test compound prior to the irradiative challenge. Fifty-five compounds were screened. Of these, four compounds gave good evidence of protective efficacy (90-100 percent survival). In further studies, one candidate drug, WR 249914, an amidinium compound, gave evidence of protection after oral administration: 80 percent survival at 600 mg/kg. Other test procedures under development include a technique utilizing the enzymes glutathione peroxidase and glutathione-S-transferase. These enzymes have been prepared in gram quantities and partially purified (25- and 15-fold, respectively) and are in process of further purification. Special evaluations for neutron protection and for protection of lung and central nervous system are being conducted.

FUTURE OBJECTIVES:

Efforts will continue to develop radioprotectant drugs or formulations with oral effectiveness for battlefield use. By synthesis, analogs of compounds with interesting protective activity will be produced for testing. Efficacy will be determined by primary screening and further studies. Development of test systems will be pursued to enable the examination of performance of candidate drugs as protectants in larger animals, the ability to protect specific organs (such as lung, bone marrow, central nervous system and gut), the protection against radiation of different energies and characteristics (gamma rays, x-rays and neutrons) and elucidation of mechanisms of action of radiation protectants.

AVAILABLE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 305983	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM
	A. New	U	U		CX	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63751A	3M463751D993	CA	064
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS			

11. TITLE (Precede with Security Classification Code)
(U) Atropine Metabolism and Multidose Autoinjector

12. SUBJECT AREAS
0615 Pharmacology 0616 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
85 01	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	0.0	00
85	1.0	66

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, D.C. 20307-5100	b. ADDRESS Division of Medicine Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR SMALLRIDGE, R C
d. TELEPHONE NUMBER (include area code) (202) 576-3551	d. TELEPHONE NUMBER (include area code) (202) 576-3014
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) UMSTOTT, C g. NAME OF ASSOCIATE INVESTIGATOR (if available)

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Pharmacokinetics; (U) RAM V
(U) Atropine; (U) Drug Metabolism; (U) Human Volunteers; (U) Auto-injector

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To determine the pharmacokinetics of atropine sulfate after administration by the Mark II Auto-injector. To ascertain if atropine absorption differs when administered by the Mark II versus the Mark I Auto-injector. This research is of military importance.

24. (U) Atropine blood levels will be measured by radioimmunoassay to support a USAMRDC human volunteer study.

25. (U) None.

DD FORM 1 MAR 83 1498

EDITION OF MAR 68 IS OBSOLETE

PROJECT 3M463764D995

MEDICAL CHEMICAL DEFENSE LIFE SUPPORT MATERIEL

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY		1. AGENCY ACCESSION DA 303187	2. DATE OF SUMMARY 84 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR) 636	
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX
9. LEVEL OF SUM A. WORK UNIT					

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63764A	3M463764D995	AB	70 WWML
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CARDS			

11. TITLE (Precede with Security Classification Code)
(U) Preclinical Studies of Anti-Chemical Warfare Drugs

12. SUBJECT AREAS
0615 Pharmacology 0620 Toxicology 0605 Clinical Medicine

13. START DATE 82 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS (In thousands)
84	4.0	450
85	4.0	497

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS (include zip code) Washington, DC 20307-5100	b. ADDRESS Division of Experimental Therapeutics Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR HEIFFER, M H
d. TELEPHONE NUMBER (include area code) (202)-576-3551	d. TELEPHONE NUMBER (include area code) (301)-427-5393
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR (if available) SCHROEDER, A g. NAME OF ASSOCIATE INVESTIGATOR (if available) CHUNG, H

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Pharmacodynamics; (U) Antidotes; (U) Toxicity; (U) Pharmacokinetics; (U) Quantitation Methodology; (U) Formulation; (U) Metabolism; (U) RA I

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) The technical objectives of this work unit are to obtain the necessary information for chemical warfare agent antidotes to support a Notice of Claimed Investigational Exemption for a New Drug (IND). The antidotes will be developed for the defense of military personnel on an integrated chemical/nuclear/conventional battlefield.

24. (U) A highly integrated, multidisciplinary effort is required to coordinate the extramural and intramural studies necessary to develop candidate chemical warfare agent antidotes. The actual studies performed are dictated by scientific rationale and existing federal regulations to include the completion of efficacy and toxicity studies, formulation development, pharmacokinetic and metabolism studies.

25. (U) 83 10 - 84 09 Two candidate nerve agent antidotes, WR 249,943 (MMB4) and WR 249,655 (HI-6), continue to undergo accelerated and chronic stability studies. These studies indicate that stability is similar to 2-PAM chloride at pH ranges of 3-3.5 and preliminary data suggests that both compounds are more stable in concentrated solutions. WR 2823, a potential anticyanide drug, was microencapsulated and tested for efficacy in mice. The results indicate that partial protection of the drug from acid hydrolysis caused only a small increase in efficacy. The metabolism of a second drug, WR 6026, which may be useful in cyanide poisoning, continues to be studied in isolated hepatocytes and microsomes. Studies of IM toxicity of WR 2823, sodium nitrate and sodium thiosulfate, have begun. Other candidate drugs with similar properties are also being studied as candidates for anticyanide therapy. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

DD FORM 1498 83 MAR

EDITION OF MAR 68 IS OBSOLETE.

Project 3M463764D995 MEDICAL CHEMICAL DEFENSE LIFE SUPPORT MATERIEL

Work Unit 070 Preclinical Studies of Anti-Chemical Warfare Drugs

Investigators:
Principal: Melvin H. Heiffer, Ph.D.
Associate: CPT A. Schroeder, MSC, CPT A. Theoharides, MSC, CPT J. Anders, MSC, H. Chung, Ph.D., J. Karle, Ph.D.
Assistant: SP4 V. Melendez, SP4 H. Velazquez, J. Bartosevich, J. DiGiovanni, SP4 M.H. Smyth, PFC M.M. Kim

1. Description.

The development of antidotes against the various chemical warfare agents in the hands of threat nations requires a highly integrated, multidisciplinary approach spanning a broad spectrum of preclinical and clinical pharmacological studies. The ultimate goal of these studies is to obtain the necessary information to support the granting of a Notice of Claimed Investigational Exemption for a New Drug (IND) by the Food and Drug Administration (FDA) for each candidate antidote.

2. Progress.

Stability studies on the oxime nerve agent antidotes WR 249,943 (MMB-4) and WR 249,555 (HI-6) continue using both accelerated and long-term protocols. Initial results indicate that the stability of these two compounds is similar to 2-PAM-Cl over the pH range 2.5 to 6.0, and their stability appears to be enhanced at greater concentrations.

In other studies, thiosulfate is being re-examined with a view toward improving and expanding its utility as an antidote to cyanide poisoning. Work is in progress to adapt analytical methods for use in animal studies to determine the lowest effective i.v. dose. Another potential anticyanide drug, WR 2823, has been microencapsulated and tested for oral efficacy in mice. Partial protection of the drug from stomach acid hydrolysis has been achieved resulting in a small increase in efficacy. Toxicity studies of this drug, sodium nitrate, and sodium thiosulfate utilizing the IM route have begun. The candidate methemoglobin-forming drug WR 6026 has been shown to have prophylactic anticyanide activity and is presently under study in isolated hepatocytes and microsomes. A method of quantitating this drug in biological fluids and tissues has been developed and is being used to collect pharmacokinetic and metabolism information. Other candidate anticyanide drugs are presently undergoing testing for efficacy.

3. Future Work.

The stability studies on the oximes continues pending the results of efficacy studies. Efforts will continue to increase the sensitivity of the thiosulfate analysis procedures for future studies in model animal systems. This work will also support IM efficacy studies. Development of WR 6026 as a pretreatment anticyanide drug will continue.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION DA 303186	2. DATE OF SUMMARY 84 10 01	*REPORT CONTROL SYMBOL* *DD-DR&E(AR) 636*
3. DATE PREV SUM'RY 83 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING	8. DISB'N INSTR'N CX
9. LEVEL OF SUM A. WORK UNIT					

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63764A	3M463764D995	AC	071 WWMK
b. [illegible]				
c. [illegible]	CARDS			

11. TITLE *(Precede with Security Classification Code)*
(U) Preclinical Studies of Antiradiation Drugs

12. SUBJECT AREAS
0615 Pharmacology 0618 Radiobiology 0620 Toxicology 0605 Clinical Medicine

13. START DATE 82 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING ORGANIZATION DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT	
a. DATE EFFECTIVE	EXPIRATION
b. CONTRACT/GRANT NUMBER	
c. TYPE	d. AMOUNT
e. KIND OF AWARD	f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	3.0	371
85	3.0	402

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research
b. ADDRESS *(include zip code)* Washington, DC 20307-5100	b. ADDRESS Division of Experimental Therapeutics Washington, DC 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, F H JR	c. NAME OF PRINCIPAL INVESTIGATOR HEIFFER, M H
d. TELEPHONE NUMBER *(include area code)* (202)-576-3551	d. TELEPHONE NUMBER *(include area code)* (301)-427-5393
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* FLECKENSTEIN, L g. NAME OF ASSOCIATE INVESTIGATOR *(if available)* RIDDER, W E

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Pharmacodynamics; (U) Radioprotectants; (U) Toxicity (U) Pharmacokinetics; (U) Quantitation Methodology; (U) Formulation; (U) Metabolism

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)* (U) [illegible]

23. (U) The technical objective of this work unit is to obtain the necessary information to support a Notice of Claimed Investigational Exemption for a New Drug (IND) for antiradiation agents being developed for defense of military personnel or an integrated chemical/nuclear/conventional battlefield.

24. (U) A highly integrated, multidisciplinary effort is required to coordinate the extramural and intramural studies necessary to develop candidate antiradiation agents. The actual studies performed are dictated by scientific rationale and existing federal regulations to include the completion of efficacy and toxicity studies, formulation development, as well as pharmacokinetic and metabolic studies.

25. (U) 83 10 - 84 09 Analytical procedures for HPLC measurement of the radioprotectant drug, WR 1065, are nearing completion. The drug is rapidly degraded during analysis. Formulation of the radioprotectant drug, WR 2721, into microspheres was completed and an IV pharmacokinetic study in dogs was completed. The oral microencapsulated WR 2721 did not obviate vomiting in dogs, therefore, pharmacokinetic studies in the rhesus monkey, both intravenously and orally, were instituted. Pharmacokinetic evaluation of WR 1065 in the rhesus monkey was begun and will be compared with the pharmacokinetic evaluation of WR 2721 when the work is completed. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 83 - 30 Sep 84.

Project 3M463764D995 MEDICAL CHEMICAL DEFENSE LIFE SUPPORT MATERIEL

Work Unit 071 Preclinical Studies of Antiradiation Drugs

Investigators:
Principal: Melvin H. Heiffer, Ph.D.
Associate: LTC W.E. Ridder, VC, L. Fleckenstein, Pharm.D.
Assistant: SP4 E. Stonecipher

1. Description.

The technical objective of this work is to obtain the necessary information to support a Notice of Claimed Investigational Exemption for a New Drug (IND) for antiradiation drugs to protect military personnel from the effects of nuclear weapons on the integrated (conventional, nuclear, chemical) battlefield.

2. Progress.

Development of acceptable analytical procedures to measure WR 1065 are nearing completion. This compound is the principal metabolite of the radioprotector, WR 2721. Preliminary results indicate that this metabolite is rapidly removed from plasma by disulfide formation with endogenous thiols. This may explain the short half-life of WR 1065 seen when it was administered i.v. to beagle dogs in preliminary pharmacokinetic studies. Following oral administration of the phosphorylated prodrug WR 2721, formulated in microspheres, peak levels of WR 1065 occur 30 to 40 minutes after dosing. The drug then disappears rapidly from plasma. Since oral dosing of WR 2721 formulations consistently produced vomiting in the dog, oral pharmacokinetic studies have been instituted in the rhesus monkey.

Work has been started to solve some of the problems noted above. Analytical procedures are being developed to measure the symmetrical disulfide of WR 1065. Methods to release bound WR 1065 from endogenous material are being explored.

3. Future Work.

Pharmacokinetic analysis of microencapsulated WR 2721 administered orally to the monkey will continue. A pharmacokinetic study of WR 1065 in monkey plasma will soon be initiated. The results of these studies will be compared to provide an evaluation of dosage regimen.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 305984	84 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
	A. New	U	U		CX	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63764A	3M463764D995	AB	072 WWJ9
b. CONTRIBUTING				
c. XXXXXXXXXXX	CARDS			

11. TITLE *(Precede with Security Classification Code)*
(U) Effects of Chemical Defense Medical Interventions on Military Performance

12. SUBJECT AREAS
0510 Psychology, 0615 Pharmacology, 0616 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
84 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE
EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE
d. AMOUNT
e. KIND OF AWARD
f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
84	0.0	00
85	1.0	388

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME Walter Reed Army Institute of Research	a. NAME Walter Reed Army Institute of Research Division of Neuropsychiatry
b. ADDRESS *(include zip code)* Washington, D.C. 20307-5100	b. ADDRESS Washington, D.C. 20307-5100
c. NAME OF RESPONSIBLE INDIVIDUAL TOP, FH JR	c. NAME OF PRINCIPAL INVESTIGATOR HEGGE, F W
d. TELEPHONE NUMBER *(include area code)* 202 - 576-3551	d. TELEPHONE NUMBER *(include area code)* 301 - 427-5653
21. GENERAL USE FINA MILITARY/CIVILIAN APPLICATION: H	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)*
(U) Medical Chemical Defense; (U) Performance Assessment; (U) Military Performance (U)RAM V

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Assess the military performance impacts of the use of newly developed chemical defense drugs for medical intervention. Manage and administer the work of the tri-service Joint Working Group on Drug Dependent Degradation in Military Performance (JWGD3 MILPERF).

24. (U) Develop a Performance Assessment System (PAS) that is responsive to both the military task performance requirements of selected operational systems and the pharmacological actions of candidate drugs. Use PAS components to assay performance effects in an integrated series of evaluations that begin with early human clinical trials and extend to evaluation of drug/performance interactions with environmental and situational stressors. Data are used in models of systems to derive specific, testable predictions of the impact of candidate drugs on system performance. Predictions are validated in directed field tests and used to develop doctrinal prescriptions for the use of protective drugs in chemical warfare operations. Since this is a tri-service effort, the approach includes a substantial amount of intra-, and extra-mural contract management.

25. (U) During FY 84, operated as an unfunded requirement of the Medical Chemical Defense Program of the USA Medical Research and Development Command. Established JWGD3 MILPERF with working membership including representatives from the Army, Navy, Airforce, and the National Institute on Drug Abuse. Developed approach outlined in para. 24 above. Completed first round implementation of plan of action. More than forty (40) program work units have been undertaken in thirteen (13) major laboratories.

DD FORM 83 MAR 1498 EDITION OF MAR 68 IS OBSOLETE

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